DEVELOPMENT AND INFECTION STRATEGIES OF BARLEY LEAF RUSTS, AND INDUCTION OF INFECTION STRUCTURES IN CEREAL RUSTS

Lesley Kellock

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University of Edinburgh
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DECLARATION

This thesis has been composed by myself and the work of which it is a record has been carried out by myself. All sources of information have been specifically acknowledged by means of reference.

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I wish to express my sincere gratitude to all the people who have helped me to complete this study. In particular I would like to single out the following people whose contribution was invaluable.

I would like to especially thank Dr. Nick Read, my supervisor, for sharing with such enthusiasm his knowledge and experience, and for critically reading this thesis. I hope he recovers soon!

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DEDICATION

This thesis is dedicated to my Grandmother

When in doubt,
make a fool of yourself.
There is a microscopically thin
line between being brilliantly
creative and acting like the
most gigantic idiot on earth.
So what the hell, leap.

CYNTHIA HEIMEL

Writing is easy.
You just stare at a blank piece of
paper until drops of blood appear on
your forehead.

GENE FOWLER

ABSTRACT

In the first part of this thesis, the developmental strategies of the two contrasting barley leaf rusts, *Puccinia striiformis* f. sp. *hordei* (barley yellow rust, BYR) and *P. hordei* (barley brown rust, BBR) were compared during infection of the susceptible cultivar Golden Promise. The techniques of fluorescence microscopy and low-temperature scanning microscopy were used correlatively to provide complementary information on the temporal and spatial development of these rusts within the host. These studies revealed many differences between the two rusts as follows. (1) The germ tubes of BYR were long and unbranched whilst those of BBR were short and branched. (2) Only BBR produced appressoria. (3) The spread from the substomatal cavity into surrounding leaf tissue was delayed in BYR but not BBR. (4) BYR formed large, aseptate, invasive runner hyphae prior to reproduction after which they became septate. BBR lacked runner hyphae. (5) BYR exhibited semi-systemic growth during the colonisation of leaf tissue whilst BBR colonised leaf tissue only around the site of penetration. (6) BYR produced morphologically distinct hyphae involved in uredinial bed formation whilst BBR did not. (7) Primary uredinia were formed some distance from the site of penetration by BYR whilst in BBR, uredinia formed at the site of penetration.

In the second part of this study, quantitative comparisons were made of the contrasting infection strategies of the two rusts in their respective optimum conditions for development. This study revealed for the first time that the semi-systemic infection strategy employed by BYR was more efficient than the localised infection strategy exemplified by BBR.

The final part of the investigation involved a study of the role of contact sensing in the induction of appressorium formation by *P. hordei* and *P. graminis* f. sp. *tritici* (wheat stem rust). Polystyrene replicas of microfabricated silicon wafers, bearing precisely defined topographies, were used as artificial substrata for growing these cereal rusts *in vitro*. This study revealed, for the first time, that consistently high numbers of appressoria of these two rusts can be induced to form in response to topographical signals alone. Directional germ tube growth was also shown to be contact-mediated. It was shown that 83-86% of germ tubes differentiated over closely spaced (1.5 - 2.5 μm) ridges and troughs, but to an insignificant extent (< 3.5%) over single ridges. The inductive topographies commonly initiated a sequence of differentiation events including, infection pegs,

vesicles, primary infection hyphae and, very occasionally, haustorial mother cells. It is proposed that, *in vivo*, the inductive signal for appressorium formation is the close spacing of the cell junctions specific to the stomatal complexes of cereal leaves.

Table 1. Abbreviations used in this thesis.

BBR	barley brown rust
BYR	barley yellow rust
dpi	days post inoculation
h	hour
hpi	hours post inoculation
kV	kilovolts
LTSEM	low-temperature scanning electron microscopy
NIAB	National Institute of Agricultural Botany
ppm	parts per million
RH	relative humidity
s.e.	standard error
s.e.d.	standard error of difference between means
TEM	transmission electron microscopy
TWA	tap water agar
v/v	volume by volume
WPBS	Welsh Plant Breeding Station
WSR	wheat stem rust
w/v	weight by volume

CONTENTS

1. REVIEW OF THE LITERATURE

1.1. General introduction

Rust fungi (Subdivision Basidiomycotina, Order Uredinales) first appear in the fossil records 130-150 million years ago (Littlefield, 1981). The earliest references to rust infections are descriptions of the disease symptoms on cereals by Ancient Greeks and Romans (Arthur, 1929; Zadoks, 1985). The fungal nature of these pathogens was first recognised after the severe black rust epidemic in Italy in 1766 (Tozetti, 1952). The rusts of wheat, maize, barley and oats are now amongst the most studied of all plant diseases because of their major economic significance in agricultural productivity.

The rust fungi parasitize living tissues of growing plants and are an increasing problem as the cultivation of economically important crops becomes more intensive and extensive (Cummins & Hiratsuka, 1983). Presently two thirds of the world's total food supply is comprised of eight major cereal crops and losses equivalent to about 10% of the world grain crop can occur annually due to rust infections (Agrios, 1988). These biotrophic plant pathogens are found on both dicotyledonous and monocotyledonous plants and have complex and variable life cycles.

1.2. The rust life cycle: general aspects

One of the unique features of rusts is that there may be up to five successive functionally and morphologically different spore states within the life cycle of a single species (Fig. 1). Some rust species with a *heteroecious* life cycle need two unrelated groups of host plants to complete their life cycle and in this case pycniospores/aeciospores and urediniospores/teliospores are produced on different host species. Other species with an *autoecious* life cycle can complete their life cycle on one host plant. Only life cycles termed *macrocyclic* have all five spore stages and many rust fungi have shortened life cycles of which there are two basic types: (1) *demicyclic*, which lack the uredinial state; and (2) *microcyclic*, where the aecial and the uredinial states are absent. The *macrocyclic* and the *demicyclic* rusts may be either *heteroecious* (i.e. host alternating) or *autoecious* (i.e. non-host alternating) but the *microcyclic* rusts are essentially *autoecious*. The host of lesser economic importance in the *heteroecious* life cycle is commonly referred to as the

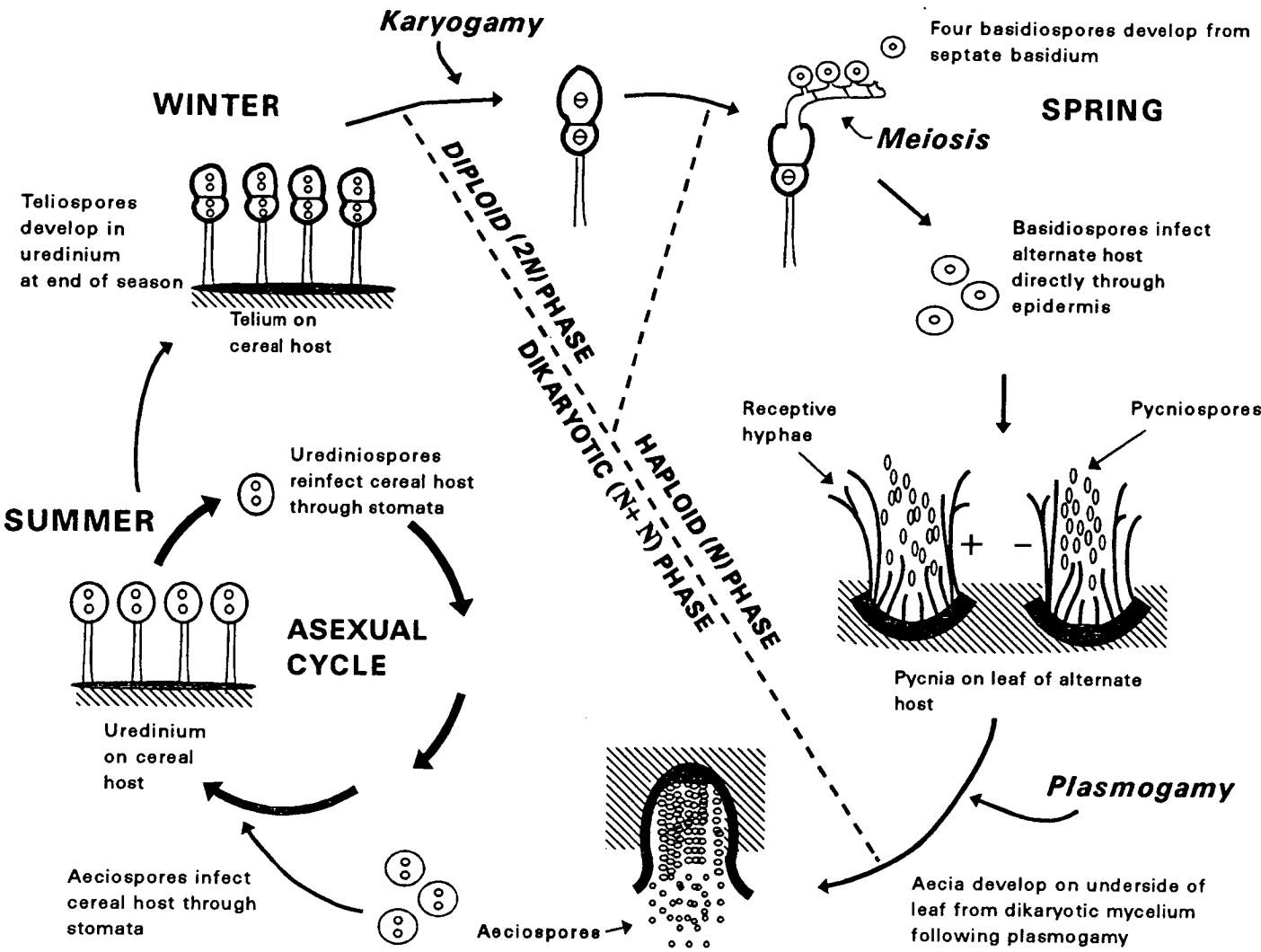


Fig. 1. Generalised life cycle of a cereal rust based on the disease cycle of *Puccinia graminis* f. sp. *tritici*. Adapted from Agrios (1988) and Roelfs *et al.* (1992).

alternate host. It is generally accepted that the *autoecious* condition and shortened life cycles are derived from *heteroecious macrocyclic* life cycles (Jackson, 1931).

Basidiospores are haploid (n) spores each borne on one of four sterigmata on a septate metabasidium developed at the end of a probasidium and stalk cell of a germinating teliospore (Fig. 1). Collectively, these structures are termed the basidium. The presence of basidiospores and basidia provides the basis for the inclusion of the rust fungi in the subdivision Basidiomycotina in which the order Uredinales is characterised by the presence of septate basidia with sterigmata after germination of the teliospore. The haploid state of the basidiospore is the result of meiosis which occurs in the basidium. The germinating basidiospore infects the alternate host or reinfects the only host depending upon the type of life cycle. From the subsequent haploid mycelium, and usually on opposite sides of the host tissue, two structures develop: (1) the pycnium, which contains pycniospores and branched receptive or flexuous hyphae; and (2) the aecial primordium, which is connected to the pycnium by the intercellular hyphae. The pycniospores and the receptive hyphae are of a single mating type (+ or -) and serve as female and male gametes which initiate further development of the aecium resulting in the formation of dikaryotic aeciospores. These non-repeating spores infect the primary host if *heteroecious*, or reinfect the same host species if *autoecious* and give rise to a dikaryotic mycelium from which uredinia and telia develop. The dikaryotic urediniospores formed in the uredinia are the 'repeating spores' of the rust life cycle and reinfect the primary host. These spores are generally of the most direct economic significance and most control measures are directed at this phase of the life cycle because their rapid build up is responsible for epidemics of cereal rusts, bean rusts, coffee rust, and other rusts over wide areas of crop monoculture (Littlefield, 1981). The urediniospores are relatively long-lived; they can survive for several weeks in the absence of the host and can be blown considerable distances by the wind.

When conditions no longer favour the uredinial phase (i.e. towards the end of the summer), infected plants eventually begin to produce teliospores in the uredinium which gradually becomes transformed into the telium. These spores serve as overwintering spores unless the rust fungus typically survives this period as dormant mycelium in host tissue. Upon germination, teliospores produce basidia and basidiospores and the (+) and the (-) nucleus in each teliospore fuses to form a temporary diploid stage. Meiosis results in two (+) and two (-) haploid nuclei which migrate into each of the four basidiospores thus completing the rust life cycle.

1.3. Taxonomy of the rusts

Unless the telial stage in the life cycle has not been found in nature, as in the so-called 'imperfect' rusts, the morphology of telia and teliospores provides the basis for the classification of the order Uredinales (Littlefield, 1981). Within this order there are traditionally three families: (1) *Pucciniaceae*, (2) *Melampsoraceae* and, (3) *Coleosporiaceae*. Of the total of around 5000 species of rusts, some 3000-4000 species occur within the genus *Puccinia* in the family *Pucciniaceae*. This genus includes many species which have cereals as their uredinial/telial host and which give rise to many important and familiar plant diseases (e.g. stem rust, stripe rust, brown rust and maize rust). Also in this family are three other economically important genera, namely: (1) *Uromyces*, whose species are common on legumes (e.g. the bean rust), (2) *Phragmidium*, which contains rusts causing diseases of members of the rose family, and (3) *Gymnosporangium*, containing rusts which parasitize juniper, apple and pear. These three genera contain approximately 600, 60 and 50 species, respectively (Littlefield, 1981). In the family *Melampsoraceae*, the genera *Melampsora* (e.g. *M. lini* causing flax rust) and *Cronartium* (e.g. *C. ribicola* causing white pine blister rust) are perhaps best known and contain about 80 and 20 species, respectively. Within many rust species identified morphologically, there is a degree of specialisation with respect to host association. To differentiate between these subspecies of rust, each subspecies is designated a different *forma specialis* ('specialised form'), commonly abbreviated as f. sp., which indicates host specificity (e.g. *P. striiformis* West. f. sp. *hordei* is the form of stripe rust which infects barley, *Hordeum* sp.).

1.4. Epidemiology of cereal rusts

The major species of cereal rusts, their hosts and favourable temperatures for disease are listed in Table 2.

1.4.1. Epidemiology of wheat rusts

The three wheat rusts are still plant diseases of major significance (Roelfs *et al.*, 1992). The most common disease is leaf or brown rust caused by *P. recondita* Rob. ex. Desm. f. sp. *tritici* and it occurs to some extent wherever wheat is grown. Yield loss generally results from a reduction in grain size and number (Stubbs *et al.*, 1986) and consequently severe losses can occur when the flag leaf is

infected (Roelfs *et al.*, 1992). The disease is controlled by resistant host genes effective locally but it is still a significant problem in some of the major wheat growing areas of the world (Roelfs *et al.*, 1992).

Potentially, the most devastating of the wheat rust diseases is stem rust (syn. black rust), caused by *P. graminis* Pers. f. sp. *tritici*. This disease is favoured by high humidity and warm temperatures of 15 to 30°C, and can cause losses of 50% in one month when conditions are favourable and 100% loss with susceptible cultivars (Roelfs *et al.*, 1992). Grain number is reduced and extensive shrivelling can occur (Littlefield, 1981; Stubbs *et al.*, 1986) as well as stem lodging after early disease onset (Roelfs *et al.*, 1992). The disease is important to North American wheat production and up to 1962 sporadic epidemics had extreme economic impact (Littlefield, 1981). Presently, the use of resistant cultivars worldwide has reduced the economic significance of the disease on crop production (Roelfs *et al.*, 1992). However, stem rust urediniospores can remain viable after long distance transport and are regularly carried by wind across North America, across from Australia to New Zealand and, at least three times in this century, across from East Africa to Australia (Roelfs *et al.*, 1992). In Europe, *P. graminis* f. sp. *tritici* spreads northwards along either the so-called West or East European Tract of *P. graminis* dispersal (Littlefield, 1981).

Yellow rust (syn. stripe rust) caused by *P. striiformis* West. f. sp. *tritici* has the lowest temperature requirements of the three wheat rust pathogens (Table 2) and causes disease on wheat grown in cooler climates (2-15°C) associated with higher elevations, cooler years or northern latitudes (Roelfs *et al.*, 1992). Stripe rust can be as damaging as stem rust causing 100% loss in extreme situations and is of major importance in East Africa, the west and the most easterly region of Asia, and in Western Europe (Roelfs *et al.*, 1992). In the Netherlands 70% of the 1956 winter wheat crop was destroyed by yellow rust (Littlefield, 1981) and as recently as 1988 there was an epidemic of this rust in the U.K. (Bayles, Channell & Stigwood, 1989). The yield and quality of grain is affected and heavy infection results in severe grain shrivelling (Stubbs *et al.*, 1986). In contrast to stem rust (*P. graminis*), severe epidemics of yellow rust in Europe do not depend upon annual long distance dispersal of inoculum by wind. Once races are established, the good cold-tolerance of this rust means it survives well on autumn-sown and volunteer wheat and these provide the source of inoculum for the following season (Littlefield, 1981).

Table 2. The major species of cereal rusts, their hosts and optimum temperatures for disease.

Pathogen species	*Formae speciales*	Primary hosts	Alternate hosts	Optimum temp. range (°C)
Puccinia graminis	*tritici* *secalis* *avenae*	wheat, barley rye, barley oats	Barberry, Mahonia	15-30
Puccinia recondita	*tritici* *secalis*	wheat rye	Meadow-Rue, *Isopyrum*, Alkanet, Clematis	15-25
Puccinia striiformis	*tritici* *hordei*	wheat barley	unknown	5-15
Puccinia hordei		barley	Star of Bethlehem	17-20
Puccinia coronata		oats	Buckthorn	20

Adapted from McCracken & Burleigh (1962), Sharp (1965), Kochman & Brown (1975, 1976), Jones & Clifford (1983), Clifford (1985) and Roelfs *et al.* (1992).

1.4.2. Epidemiology of barley leaf rusts

Over the last 10-15 years the cultivation of barley has intensified in cool, temperate regions resulting in a subsequent increase in the barley leaf rusts, namely yellow rust (syn. stripe rust) caused by *Puccinia striiformis* f. sp. *hordei* and brown rust (syn. leaf or dwarf rust) caused by *P. hordei*. Of the two diseases, barley brown rust is generally more widespread and important (Clifford, 1988) although in cooler growing seasons barley yellow rust can be of greater significance (Zadoks, 1988) and can reach epidemic proportions under favourable cool and moist conditions (Stubbs, 1985; Dubin & Stubbs, 1986). Even small but regular infections by *P. striiformis* f. sp. *hordei* can impair the malting quality of barley (Zadoks, 1988). The disease occurs in Europe, the Middle East and in mountainous regions of Asia. In Tibet, yellow rust on barley has historically been an important disease where wheat is a minor crop (Roelfs *et al.*, 1992) and this disease is the main yield constraint of barley in the cooler areas of Northern India (Kumar *et al.*, 1988). In North Western Europe, an epidemic of barley yellow rust in 1961 caused yield losses of up to 80% in the commercial barley crop (Dantuma, 1964). *P. striiformis* f. sp. *hordei* was introduced into South America in 1975 and later spread across most of that continent (Dubin & Stubbs, 1986). The disease is a problem in South America where cultivated barley, as well as wild barleys which serve as collateral hosts, are present all year (Dubin & Stubbs, 1986). More recently, barley yellow rust has spread to North America from Mexico (Roelfs & Huerta-Espino, 1992). At present, European barley cultivars are highly resistant to the form of the fungus which infects wheat, namely *P. striiformis* f. sp. *tritici*, and many races virulent to wheat have evolved. This contrasts with barley yellow rust in which virulent races have been slower to appear, there being only three to date in the UK, the last having been reported in 1983 (Bayles & Thomas, 1983; Meadway & Stigwood, 1992). This is because plant breeders have infrequently used major gene specific resistance against yellow rust in barley (Gair *et al.*, 1978). However, it is likely that barley yellow rust will acquire greater importance, and increase in pathogenicity, if the acreage of winter barley continues to increase (Stubbs, 1985).

The causal agent of brown rust of barley, *Puccinia hordei* Otth., is widely distributed wherever barley is grown and can cause yield losses of up to 20% (Hoffmann & Schmutterer, 1983). Losses occur primarily from a reduction in grain number and shrivelled grain (Stubbs *et al.*, 1986). Winter barley infected by *P. hordei* has a smaller green leaf area and is more susceptible to damage at low temperatures (McAinsh *et al.*, 1990). The disease commonly occurs at damaging

levels in North America, Argentina, New Zealand and North West Europe and intensification of barley cultivation in the latter region has resulted in a considerable increase in the incidence of the disease in the last 10-15 years (Clifford, 1988). In 1970 and 1971 there were epidemics of this rust in the U.K. on susceptible spring barley cultivars (Clifford, 1985) and during 1989 and 1990 brown rust occurred at severe levels in winter barley in England and Wales (Polley & Slough, 1992).

1.4.3. Epidemiology of oat rusts

Crown rust of oats, caused by *P. coronata* Cda. f. sp. *avenae* F. et L. is the most important disease of this cereal world-wide (Jones & Clifford, 1983; Simons, 1985). The rust derives its name from the appearance of the short apical projections on the upper cell of the teliospore. Crown rust has caused significant losses in Australia and in north-west and south-east Europe (Jones & Clifford, 1983; Simons, 1985) and is widespread in North America along the 'Puccinia path' which extends from central Mexico to the Prairie Provinces of Canada. The fungus has adapted to a wide temperature range with an optimum of around 25°C (Jones & Clifford, 1983). Infection can result in severe losses of both grain yield and quality and a loss of winter hardiness where winter oats are grown (Simons, 1985).

The fungus does not affect other cereal crops (Table 2) but it occurs on some grasses. It is heteroecious and alternates with buckthorn (*Rhamnus catharticus* L.) (Table 2) and the wide distribution of this alternate host contributes in part to the great diversity in physiological races of this pathogen; in some oat producing areas such as Africa, South America and Mexico, the inability to keep up with the occurrence of new races has contributed to a decline in oat production (Zillinsky, 1983).

Typical crown rust symptoms are bright orange uredinial pustules on leaves, sheaths and floral parts. The accompanying orange spores are the major source of primary inoculum due to the ability of the fungus to overwinter as mycelium or uredinial infections. The only limiting factor to survival in this case is the reduction in winter hardiness of the infected host (Jones & Clifford, 1983). The fungus also survives winter as teliospores on host-plant debris and aeciospore infections from buckthorn shrubs and hedges are responsible for severe local epidemics of crown rust in northern climates (Jones & Clifford, 1983; Simons, 1985).

Oats are also attacked by *P. graminis* f. sp. *avenae*, resulting in oat stem rust, which periodically has caused severe crop losses. It is a disease of continental climates of North America, Australia, Central and Eastern Europe and the Mediterranean basin (Roderick *et al.*, 1994). Generally, however, yield losses are negligible as in the USA in 1992 (Roelfs, 1993). The majority of oat cultivars grown are susceptible to the most pathogenic *P. graminis* f. sp. *avenae* race (Roelfs, 1993) and the lack of an oat rust epidemic is possibly due to a late onset of disease (Roelfs *et al.*, 1993). Interestingly, both crown rust and oat stem rust host-pathogen systems have co-evolved in the same regions for a long time yet host genes for resistance to oat stem rust are rare compared with those for resistance to *P. coronata* (Martens, 1985).

1.4.4. Epidemiology of rye rusts

The rusts of rye, namely rye leaf rust (caused by *P. recondita* f. sp. *secalis*) and rye stem rust (caused by *P. graminis* f. sp. *secalis*), are only minor diseases worldwide. This is partly because rye is a cross-pollinated cereal which results in some diversity for resistance. It is also because rye leaves normally senesce at an earlier growth stage than other cereals, so infection needs to be severe early in the season to have a significant effect on yield (Roelfs, 1985*a*).

Historically, rye stem rust (*P. graminis* f. sp. *secalis*) has been more important in northern Europe (Roelfs, 1985*b*). It also occurs in Australia and the United States but causes little damage. A severe epidemic occurred in Brazil in 1982 where old land cultivars of rye were grown (Roelfs, 1985*b*).

Rye leaf rust *(P. recondita* f. sp. *secalis*) occurs in Australia (Luig, 1985) and in the United States but causes only local crop losses (Roelfs, 1985*a*).

1.5. Life cycles and disease cycles

Generally, where environmental conditions are favourable, the cereal rust fungi reproduce by continual production of the asexual uredinial stage. Within this urediniospore phase, the infection cycle can be broken down into clearly defined stages which progress with time: (1) the *incubation period* which consists of a series of infection events (spore deposition, germination, penetration and growth within host tissues, and symptom expression), (2) the *latent period* which encompasses these initial infection events and also includes the next stage, that of the eruption of the first uredinia through the host surface, and (3) the

infectious period which covers the time of spore production and dispersal until the uredinia are non-functional (Teng & Close, 1978). Rusts produce a succession of specialized infection structures (a germ tube, an appressorium, infection peg, substomatal vesicle, infection hyphae, haustorial mother cells and haustoria) during the early stages of infection from a urediniospore. With the exception of haustoria, all of these infection structures are extracellular (Fig. 2).

In sections 1.5.1. to 1.5.3., more detailed descriptions are given of the life cycles and disease cycles of the cereal rusts investigated in this thesis.

1.5.1. *Puccinia graminis*

P. graminis causes stem (black) rust of cereals and its life cycle is of the complete two-host, five spore type (i.e. it is a macrocyclic heteroecious rust, Fig. 1). It produces pycnia and aecia on barberry and mahonia and uredinia and telia on cereals and grasses (Table 2). The rust is more familiar in the form which attacks wheat, namely *P. graminis* f. sp. *tritici*, and it provides the oldest example of attempts at control by eradication of an alternate host, in this case barberry, in 1660 in France (Littlefield, 1981). Barberry eradication has now been carried out in many parts of the world in an attempt to prevent the major source of variation in the virulence and aggressiveness of the stem rust pathogen which made breeding for resistance difficult (Roelfs *et al.*, 1992). Now the most common source of the stem rust disease world-wide is the asexual disease cycle (Roelfs *et al.*, 1992).

The uredinia of *P. graminis* occur on leaves, stems, leaf sheaths and parts of the cereal flower. The pathogen can overwinter in this uredinial state on cereal crops or on volunteer plants but, failing this, urediniospores can be introduced into the summer crop by long-distance transport by air dispersal. In the UK, stem rust is rarely a problem because the urediniospores arrive late in the season from south-west Europe and north Africa (Jones & Clifford, 1983). Free water is required for urediniospore germination and, after the formation of appressoria, light is needed for penetration via stomata (a process which takes around three hours as the temperature rises from 18 to 30 °C), infection development and sporulation (Roelfs *et al.*, 1992). Given optimum conditions (Table 2), this asexual infection cycle can repeat every 14 days (Roelfs *et al.*, 1992). Initially the uredinia appear as red-brown pustules surrounded by the epidermis which is conspicuously stripped back. Towards the end of the season, or when conditions are unfavourable, abundant numbers of black teliospores are produced and this condition gives rise to the alternate name, black stem rust, of this disease.

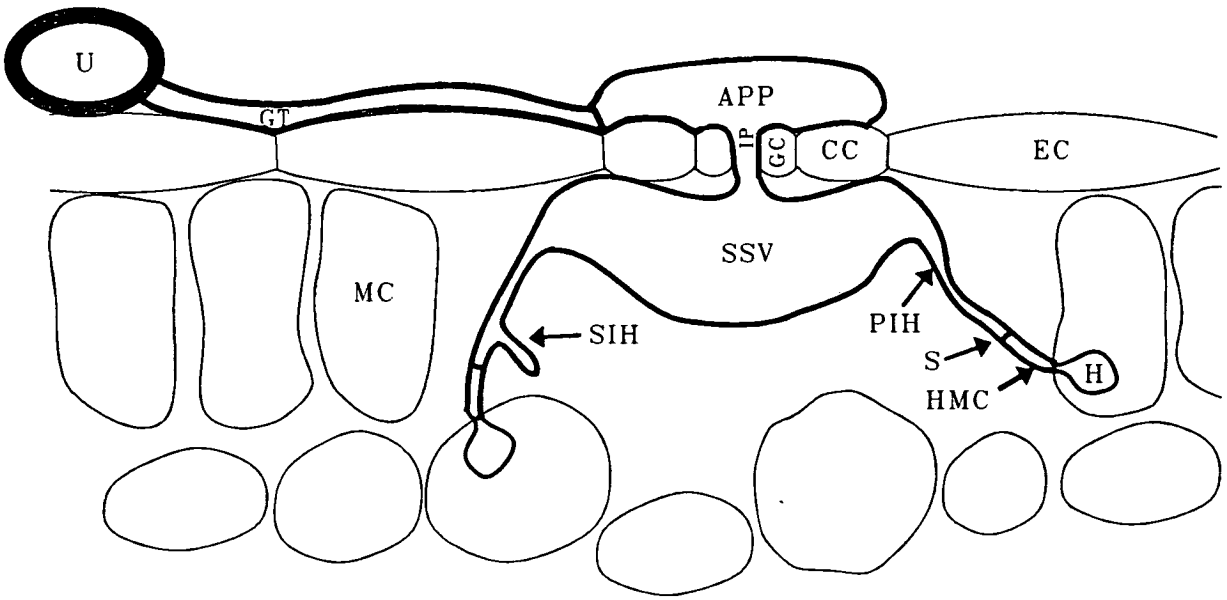


Fig. 2. Diagrammatic representation of infection structures of *P. graminis*. (Not to scale) Adapted from Allen (1923*a*,*b*), Niks (1986*b*) and Lennox & Rijkenberg (1989). Note that the SSV would normally be orientated parallel to the long axis of the stoma.

Abbreviations: APP, appressorium; CC, companion cell; EC, epidermal cell; GC, guard cell; GT, germ tube; H, haustorium; HMC, haustorial mother cell; IP, infection peg; MC, mesophyll cell; PIH, primary infection hypha; S, septum; SIH, secondary infection hypha; SSV, substomatal vesicle; U, urediniospore.

The most detailed description to date of the morphology of infection structures of *P. graminis* remains that of Allen (1923*a*,*b*) where she described the appearance of the fungal and host cells during infection of both resistant and susceptible wheat hosts. More recently, the infection process of *P. graminis* within the leaf of hosts and non-hosts has been imaged by scanning electron microscopy (Lewis & Day, 1972; Lennox & Rijkenberg, 1989). In both studies, in the susceptible host the germinated urediniospore gave rise to a germ tube exhibiting growth perpendicular to the long axis of the leaf. Appressorium formation occurred over a stoma and penetration was via a flattened infection peg which gave rise to an elongated, apparently aseptate, substomatal vesicle orientated parallel to the long axis of the stomatal opening. Primary infection hyphae then formed with terminal haustorial mother cells (HMCs) delimited by a septum. Haustoria developed from each HMC. Secondary intercellular infection hyphae formed as branches from the primary infection hyphae and these in turn also developed terminal HMCs and haustoria. Allen (1923*b*) further described how all available intercellular spaces within the host leaf became filled with septate mycelium and this proliferation of hyphae gave rise to the uredinial bed and ultimately, urediniospores. The ultrastructure of some of these fungal cells, including a comprehensive description of the haustorium, in *P. graminis*, has also been provided (reviewed by: Littlefield & Heath, 1979; Harder & Chong, 1991).

1.5.2. *Puccinia striiformis*

Puccinia striiformis possesses a short life cycle with one host only. The life cycle is normally comprised only of uredinial and telial states. Basidiospores, however, can also be formed (Wright & Lennard, 1978) although no alternate aecial host has been identified. *P. striiformis* is therefore dependent upon the cereal host for survival and the rust overwinters and oversummers in the uredinial state, often as dormant mycelium.

The disease appears as scattered yellow uredinial pustules on young leaf blades or as stripes between veins on adult leaves. At the first appearance of the disease, usually in the early spring, there may be a rapid build up of inoculum. New cycles of the disease may be caused by these local urediniospores. Infection by inoculum dispersal over long distance may also occur but is often considered of lesser importance; it is known that yellow rust spores are very sensitive to UV (ultraviolet) light which would have a deleterious effect on their long distance survival (Maddison & Manners, 1972). Later on in the summer, infection is often curtailed by hot, dry weather. This is because 100% humidity, usually in the form

of dew, is necessary for urediniospore germination, and dew formation is often associated with relatively low temperatures. In barley, if the infection persists, and thus becomes severe, pustules will appear on the awns and this has an effect on grain size and quality or can even cause total loss of yield. When conditions are no longer favourable for sporulation, or at the end of the growing season, the rust lesions often contain black telia but the subsequent teliospores serve no known purpose.

Yellow rust is exceptional in that it is favoured by relatively low temperatures (Table 2) and is predominant in cool, moist weather. It is therefore common in some years but rare in others. Unlike other rusts it is extremely sensitive to environmental conditions. Many of the environmental parameters favourable for yellow rust development have only been reported for wheat yellow rust. However, it is likely that the critical phases of barley yellow rust development will have similar requirements. Indeed, it is known that relatively small differences in both pre- and post-inoculation temperatures can significantly affect infection levels of barley yellow rust (Kellock & Lennard, 1982).

The optimum temperature for urediniospore germination of yellow rust has been reported as about 10°C (Manners, 1950; Osman-Ghani & Manners, 1983), between 2 and 5°C (McCracken & Burleigh, 1962) and 7°C (Sharp, 1965). Spores of yellow rust produced in cool, moist conditions often germinate better than those produced under warm, dry conditions (Gopalan & Manners, 1984). The latent period of wheat yellow rust was found to be reduced by increasing temperature up to 20°C; 7-15°C was found optimal (McGregor & Manners, 1985). Light intensity also has a significant effect on yellow rust development: increased light increased the number of pustules in a unit area and the rate of sporulation on wheat (McGregor & Manners, 1985).

Yellow rust is unique with respect to other cereal rusts in that extensive colonization can occur from a single penetration site. This systemic nature of the fungus means that more pustules arise from growth within the leaf than from re-infection (McGregor & Manners, 1985).

The development of infection structures by *P. glumarum* (Schm.) Erikss. u. Henn. (= *P. striiformis*) was recorded by Allen (1928). Given the limitations of equipment in those early days her light microscopy studies are remarkable for their detail. The meticulous drawings and observations of infection of wheat and a *Bromus* species remain the classic reference for further study of the histology of this fungus. Among her many observations, she noted that entry was via stomata without the formation of an appressorium and that this was then followed by a

protracted vegetative phase. After substomatal vesicle formation, infection hyphae did not appear until some time after penetration. She also stated that the fungus was predominantly aseptate and that, with the exception of the delimitation of haustorial mother cells, the appearance of septa was a clear indication of the beginning of reproductive activities. Her descriptions provide some notable contrasts to those of *P. graminis* namely: the absence of appressoria; the delay in the formation of infection hyphae; and the initially aseptate nature of the infection hyphae. More recent cytological work on yellow rust has supported Allen's findings. With respect to wheat yellow rust, infection events in susceptible and resistant reactions have been visualized by light, fluorescence and transmission electron microscopy (Mares & Cousen, 1977; Mares, 1979; Cartwright & Russell, 1981); and nuclei and mitosis have been examined in germ tubes and infecting hyphae (Little & Manners, 1969; Goddard, 1976; Wright & Lennard, 1978; Wright, Lennard & Denham, 1978). Barley yellow rust has also been examined by ambient-temperature scanning electron microscopy (Schmidt *et al.*, 1985; Opel *et al.*, 1986*a*,*b*), and low-temperature scanning electron microscopy (LTSEM) (Read, 1991; Read *et al.*, 1992) and fluorescence microscopy (Opel *et al.*, 1986*a*,*b*).

1.5.3. *Puccinia hordei*

P. hordei is a macrocyclic and heteroecious rust like *P. graminis* (see section 1.5.1.). The uredinial and telial stages occur on cultivated and wild species of barley; pycnia and aecia are found on three species in the Liliaceae. The alternate host is essential for the survival of the pathogen in Israel but is unimportant in Europe. The fungus can survive as the uredinial state under winter conditions in cool temperate regions (Clifford, 1985).

The disease appears on the leaf blade as small, round, orange-brown pustules which are often surrounded by a chlorotic halo. On the mature crop, blackish-brown telia may be found in stripes on the leaf-sheaths (Clifford, 1985). The urediniospores are dispersed by wind, and infection progresses rapidly in warm summer weather when free water, required for spore germination, is available overnight. Spring barley is particularly at risk and early infections can reduce root and shoot growth (Clifford, 1985). Epidemics generally occur later in the season with a consequent reduction in grain size and quality (Clifford, 1985).

In contrast to yellow rust the cardinal temperatures for brown rust development are relatively high (Table 2). Germination and colonization increase between 10-20°C and 5-25°C, respectively, and the time to sporulation can be as

short as 6 days at 25°C (Simpkin & Wheeler, 1974). The period of spore production is similar in the range of 10-20°C (Teng & Close, 1978).

The histology of brown rust of barley was described in some detail by D'Oliveira (1938) who produced detailed diagrams and descriptions of infection structures in susceptible and resistant host cultivars. He reported that an appressorium was always formed over a stoma before penetration and subsequent formation of a 'sausage-shaped' substomatal vesicle was followed by production of infection hyphae and a 'primary septum' in the vesicle. In resistant hosts, necrosis first appeared at this stage. In a susceptible reaction, growth of infection hyphae progressed by branching and haustoria were produced of various shapes and sizes. The colony expanded to a semi-spherical shape in which a subepidermal mass of hyphae was formed giving rise to the uredinial pustule bed. As spore production declined in the uredinium in the centre, secondary pustules were frequently formed.

More recent studies have not examined the histology of this pathogen in such extensive detail and most have concentrated on host resistance mechanisms (Clifford, 1972; Reynolds, 1975; Clifford & Roderick, 1978; Niks, 1981, 1982; Niks & Kuiper, 1983; Niks, 1983*a*,*b*; Niks, 1986*a*; Parlevliet & Kievit, 1986; Niks, 1990; Rubiales *et al.*, 1993). This is mainly because partial resistance (PR) and major gene resistance (hypersensitive response) are two options available to control this pathogen in the field. Other cytological work on brown rust has been used to: localize and measure the frequency of haustoria in developing colonies (Kneale & Farrar, 1985); correlate photosynthesis rates with infection (Scholes & Farrar, 1987); and examine the influence on infection of a second rust species (Niks, 1989).

1.6. Role of contact sensing during infection by rusts

The germ tubes of rust fungi possess a highly sophisticated capability for touch (contact) sensing of the host surface and this plays an important role in achieving successful infection of a host plant. During the pre-penetration phase, in particular, rusts respond in a variety of specific ways to touch. The best characterised contact-mediated response has been the induction of appressorium differentiation over stomata on bean leaves by *Uromyces appendiculatus*. Essential evidence to demonstrate that any response on a host plant is, indeed, contact-mediated requires the response to be mimicked on an artificial substratum in the

absence of chemical signals. Various substrata bearing topographical signals have been used to induce appressoria in a wide variety of rust species. However, the best artificial substrata for these studies have been polystyrene replicas of microfabricated silicon wafers because they are highly precise and reproducible in their three-dimensional topography and dimensions (Read *et al.*, 1992).

Wafer replicas were first used by Hoch *et al.*, (1987) to analyse rust contact sensing. These workers demonstrated that *U. appendiculatus* is optimally induced to differentiate appressoria over steps with a height of 0.5 μm. This topographical signal was closely correlated with the size of the guard cell lip (or "ledge") of the host plant *Phaseolus vulgaris* (Hoch *et al.*, 1987; Allen *et al.*, 1991*b*; Terhune *et al.*, 1991). In a survey of 27 different rust species, Allen *et al.* (1991*a*) subsequently found that most species responded to single ridges spaced 60 μm apart by forming appressoria, but the patterns of response to different ridge heights varied significantly between species. In addition, a notable group of rusts were observed which did not differentiate over single ridges. They included a number of economically important cereal rusts (*P. graminis* f. sp. *tritici*, *P. g.* f. sp. *avenae*, and *P. coronata*). On scratched, artificial substrata, appressorium formation by *P. graminis* f. sp. *tritici* (the most studied cereal rust), has been very variable ranging from 3% to 68.5% differentiation (Staples *et al.*, 1983; Allen *et al.*, 1991*a*). *In vivo* 90% of germ tubes of this rust differentiate appressoria on encountering wheat leaf stomata (Collins, T. & Read, N.D. unpublished).

There has thus been considerable controversy as to what stimulates P. *graminis* f. sp. *tritici* to differentiate appressoria so precisely and efficiently over cereal leaf stomata. Because of the inconsistent and often very poor results obtained with scratched surfaces and wafer replicas, some workers have suggested a role for chemical signalling in appressorium induction in P. *graminis* f. sp. *tritici* and related cereal rust species. However, the significance of chemicals inducing rust appressoria is not clear (Read *et al.*, 1992). Nevertheless, there is some evidence that the chemical environment around stomata may provide important cues or modulate contact-mediated appressorium formation (Grambow & Reisener, 1976; Grambow, 1977, 1978; Grambow & Riedel, 1977; Grambow & Grambow, 1978).

Another contact-mediated response exhibited by rust germ tubes is directional growth at right angles to epidermal cell junctions. This phenomenon has been demonstrated in many rust species growing on a variety of different hosts (Maheshwari & Hildebrandt, 1967; Lewis & Day, 1972; Wynn, 1976; Pring, 1980; Wynn & Staples, 1981; Mendgen, 1982; Hoch *et al.*, 1987; Lennox &

Rijkenberg, 1989; Allen *et al.*, 1991*a*; Read *et al*, 1992; Rubiales & Niks, 1992). This directional growth response has also been mimicked on a range of artificial substrata indicating that it is contact-mediated (Maheshwari & Hildebrandt, 1967; Dickinson, 1969; Hoch *et al.*, 1987; Read *et al.*, 1992). On graminaceous hosts, growth at right angles to the epidermal cell junctions increases the probability of contact with stomata which, in this case, are arranged in staggered parallel rows (Johnson, 1934; Read *et al.*, 1992).

Other contact-mediated responses exhibited by rusts include the asymmetric cytological organisation of the germ tube relative to the substratum and localised adhesion of the rust germ tube to the contact surface. The tips of rust germ tubes often exhibit a 'nose-down' asymmetry (Dickinson, 1969; Wynn, 1976; Epstein *et al.*, 1987; Hoch *et al.*, 1987; Koch & Hoppe, 1988; Read *et al.*, 1992) and the germ tube is frequently flattened at the interface with the substratum, even to the extent of moulding itself into the features of the underlying surface (Mendgen, 1973; Bourett *et al.*, 1987, Read *et al.*, 1992). The germ tubes adhere tightly to surface waxes (Lewis & Day, 1972; Read *et al.*, 1992) and this close contact between germ tube and host surface is usually achieved by the presence of a proteinaceous extracellular matrix which binds the germ tube to the surface (Epstein *et al.*, 1985; Nicholson and Epstein, 1991; Read *et al.*, 1992).

The mechanism of contact sensing by rusts is unknown. However, there is growing evidence for a multiplicity of signal transduction components being involved (see Read *et al.*, 1992, for a detailed discussion).

1.7. Introduction to the research carried out in this thesis

The first aim of this study was to examine in detail the developmental morphology of the two markedly contrasting barley rusts, *P. striiformis* f. sp. *hordei* (yellow rust) and *P. hordei* (brown rust) on seedling leaves of the susceptible cultivar Golden Promise. This investigation represents the first in which LTSEM and fluorescence microscopy techniques have been used correlatively to provide complementary information about plant-pathogen interactions during any rust infection. Many specific developmental adaptations by each species to their two very different infection strategies were found. Amongst these it was noted that barley yellow rust is primarily aseptate before reproduction. It is suggested that this is an adaptation for its semi-systemic growth behaviour; extensive septation only occurs during reproduction. Barley brown rust, on the other hand, with its

localised type of infection strategy has a more immediate investment in reproduction and septation occurs from the beginning of infection. The fact that yellow rust successfully penetrates without the formation of an appressorium is another of several characteristics which demonstrate that this rust has an alternative, yet obviously very successful, infection strategy.

The second aim was to provide a quantitative comparative analysis of the infection strategies of these two rusts in their respective optimum conditions on Golden Promise. This study revealed, for the first time, that the semi-systemic infection strategy employed within the leaf by *P. striiformis* can be more efficient than the localised infection strategy exemplified by *P. hordei*. The measurement of spore production was found to give the most precise and reliable assessments for comparison of the relative success of each infection strategy.

The third aim was to investigate the role of contact sensing in the induction of appressorium formation in *P. hordei* and in *P. graminis* f. sp. *tritici* (wheat stem rust) where hitherto the importance of contact sensing in successful infection was either unknown (in the case of *P. hordei*) or unclear. This study shows, for the first time, that a consistently high percentage of germ tubes of the cereal rusts *P. hordei* and *P. graminis* f. sp. *tritici* can be induced to differentiate appressoria in response to topographical signals alone. The inductive topographies commonly initiate a sequence of differentiation events which result in the formation of appressoria, infection pegs, vesicles, infection hyphae and, very occasionally, haustorial mother cells. These infection structures are usually only formed inside the leaf. It is suggested that the inductive topographical signal *in vivo* is the close spacing of cell junctions associated with the stomatal complexes of cereal leaves.

2. MATERIALS AND METHODS

2.1. Growth conditions

Plants of the susceptible barley cultivar Golden Promise were grown at 16°C with a 16 h photoperiod and at 80% RH up to Feekes growth stage 12 (i.e. the complete emergence of the first true leaf (Tottman & Makepeace, 1979)). The average length of these leaves was 151.5 mm. At this stage plants were inoculated with either 0.020 g of urediniospores of *Puccinia hordei* Race octal 1653 (brown rust virulence factors - 1,2,4,6,8,9,10), obtained from WPBS, Aberystwyth, or 0.020 g of urediniospores of *P. striiformis* f. sp. *hordei* isolate 74/33 [1988] (barley yellow rust virulence factor - 1) from NIAB, Cambridge, in a settling tower 0.57 m diameter and 1.66 m high. Greased slides were placed in the base of the tower to check the inoculum distribution and density. The latter was always approximately 1000 urediniospores per cm^2. This moderate inoculum density is within the range used by others working on barley brown rust (Teng & Close, 1978; Niks, 1981; Niks & Kuiper, 1983; Casulli, 1985; Helfer, 1986) and wheat and barley yellow rust (Sharp, 1962; Tollenaar & Houston, 1966; Helfer, 1986; Rubiales & Niks, 1992). The insides of transparent, plastic domes were misted with water and placed over the plants to ensure 100% humidity. Plants inoculated with *Puccinia hordei* were kept at 15°C in the dark for the first 24 h after inoculation to encourage germination and appressorium formation (Clifford, 1985). Thereafter, the domes were removed and the plants kept at 18°C, 80% RH with a 16 h photoperiod. Plants inoculated with *P. striiformis* f. sp. *hordei* were kept at 5°C in the dark for the first 48 h after inoculation and the domes then removed and the plants grown at 14°C, 80% RH with a 16 h photoperiod (Kellock & Lennard, 1982; Osman-Ghani & Manners, 1983).

Plants of the susceptible spring wheat cultivar Armada were grown to Feekes growth stage 12 under the same conditions of day length and RH as plants of Golden Promise, but at a higher temperature of 18°C. Plants were brush-inoculated with spores of *P. graminis* f. sp. *tritici* (isolate 84 from NIAB, Cambridge) and misted domes placed over the plants. Inoculated plants were kept at 18°C in the dark for the first 3 h to encourage germination and sporeling development (Stubbs *et al.*, 1986; Roelfs *et al.*, 1992) and then subjected to a 21 h photoperiod at 22°C after which the domes were removed. Incubation conditions of 22°C, 60% RH and a 16 h photoperiod were maintained thereafter, and spores were harvested from 6 days after inoculation.

2.2. Developmental morphology studies

2.2.1. Fluorescence microscopy

Segments from the middle of infected first true leaves were removed at daily intervals after inoculation and prepared for fluorescence microscopy according to a modification of the method described by Cartwright & Russell (1981) as used by Kellock, L.J. & Lennard, J.H. (unpublished). Leaves were cleared in near boiling 200% w/v chloral hydrate solution (BDH Ltd., Dorset, England) for approximately 5 min and then washed in distilled water. They were then immersed in O.5 M NaOH for 5 min followed by rinsing in four changes of distilled water. The cleared leaves were stained in 3% (v/v) Uvitex BHT (Ciba Geigy Ltd., Manchester) in 20% glycerol (Fisons plc., Loughborough, Leicestershire) for 5 min and washed in four changes of distilled water and left overnight in the final wash. They were then rinsed for a further 24 h in a change of water before being mounted in 20% glycerol. Prepared slides were stored at 4 °C in the dark for up to two months without apparent deterioration.

Prepared leaves were examined with a Leitz Ortholux epifluorescence microscope using either of the following filter combinations: (1) 340-380 nm excitation filter, 400 nm dichroic mirror, and a 430, 460 or 490 nm long pass barrier filter; or (2) 350-460 nm excitation filter, 510 nm dichroic mirror, and 515 nm long pass barrier filter. Micrographs were recorded on either Kodak TMAX 100 or Kodak Technical Pan 2415 35 mm film.

2.2.2. Low-temperature scanning electron microscopy

Segments of first true leaves at suitable stages of infection were prepared for LTSEM using the EMscope SP2000 cryopreparation system (Beckett & Read, 1986) interfaced with a Cambridge S250 scanning electron microscope. Samples were mounted on standard EMscope LTSEM stubs using Tissue Tek O.C.T. compound (Miles laboratories Inc., Naperville, Illinois, USA) as a cryoadhesive. Depending on subsequent preparation, leaf segments were either mounted flat on the stub surface or located on edge in grooves cut into the surface of the stub (Jeffree & Read, 1991). Mounting was performed as rapidly as possible (within 1 min) to minimise specimen desiccation. The stub with 1-3 mounted leaf segments was cryofixed by plunging it into sub-cooled nitrogen under dry argon gas. Some samples were freeze-fractured whilst others were not. Freeze fracturing was performed using either a knife or blade which had been pre-cooled. Specimens

were examined either fully frozen-hydrated or partially freeze-dried after warming to -65°C to -70°C on the microscope cold stage. All material was finally sputter-coated with gold and examined below -155°C with accelerating voltages between 5 and 7 kV. Scanning electron micrographs were recorded on TMAX 100 120 roll film.

2.3 Quantitative assessments

2.3.1. Growth conditions

For measurements of germ tube lengths and colony size, whole plants were grown and infected as described above. For all other measurements, plants were grown in a "Burkard Propagator" providing spore-free conditions in a greenhouse environment. For assessments of percentage germination and penetration, 3 cm segments from the mid-region of first true leaves were removed from plants at Feekes growth stage 12 (emergence of first true leaf) and placed onto 0.25% agar containing 80 ppm benzimidazole (Sigma Chemical Co. Ltd., Poole, Dorset) in 9 cm plastic Petri dishes. The leaf segments or whole plants were inoculated and incubated as described above.

2.3.2 Quantification of infection parameters

2.3.2.1. Germination, germ tube length, penetration events and colony size

Percentage urediniospore germination (assessed from 400 urediniospores of each species) and primary germ tube lengths (mean of 40 both undifferentiated and penetrated for each species) were measured on uncleared, infected leaf segments examined 2 dpi using fluorescence microscopy after staining with Uvitex BHT. The number of successful penetration events (indicated by the presence of a vesicle) was similarly assessed at this stage by fluorescence microscopy except that cleared leaf segments were used. Eight segments for each species were analysed which represented an assessment of 452 penetration events for barley yellow rust and 525 for barley brown rust. All three experiments were performed twice. Colony size (n=20) was analysed in cleared leaf segments removed from the first true leaves of infected plants at specified intervals over a 35-day period. Two 3 cm segments from the mid region of four leaves were removed at each interval. The methods used for fluorescence microscopy, staining and clearing of leaves are those described in section 2.2.1.

2.3.2.2. Uredinial number, spore production and length of leaf infected

These parameters were recorded at 2-day intervals from the onset of symptoms until the end of the infection period:

Uredinial number. The number of actively sporulating uredinia was counted from the first true leaves of plants grown alongside those used for assessments of spore production and the length of leaf infected.

Spore production. Spores from individual leaves were collected on individual pre-weighed membrane filters (cellulose nitrate, pore size 0.45 μm, Whatman Scientific Ltd., Maidstone, Kent) using an impaction collector modified from a design by Teng & Close (1977). Spores and membranes were kept in glass Petri dishes overnight at 4 $^{\circ}$C prior to weighing in order that they fully equilibrated with the surrounding atmosphere.

Length of leaf infected. The length of the first leaf supporting actively sporulating colonies was assessed after each spore collection (see above).

The whole experiment was performed twice.

2.3.2.3. Urediniospore size and numbers

The lengths and widths of 100 hydrated urediniospores of each species were measured after placing them in distilled water. The number of urediniospores per mg of non-hydrated spores was assessed from 3.0 mg of spores of each rust suspended in a fixed volume of cottonseed oil (Sigma Chemical Co. Ltd., Poole, Dorset). The mean number of spores per ml was determined using a haemocytometer from three replicate preparations of 14 assessments for each species.

2.3.2.4. Statistical analysis

All data sets were analysed using 'Genstat', version 5.0 (Statistics Department, Rothamsted Experimental Station).

2.4. Urediniospore morphology in the hydrated and non-hydrated condition

Urediniospores collected for the analysis of spore production were examined in their non-hydrated (in cottonseed oil) or hydrated state (in distilled water) using a Reichert-Jung Polyvar photomicroscope equipped with differential interference contrast optics. Micrographs were recorded on Kodak TMAX 100 35mm film.

2.5. Wafer replica studies

2.5.1. Preparation of microfabricated silicon wafers

Silicon wafers providing highly reproducible and precise topographies were designed by Dr. N.D. Read and manufactured by the Microfabrication Unit in the Department of Electrical Engineering, University of Edinburgh. Microfabrication was achieved by photolithography (Read *et al.*, 1992). This involved initially coating flat silicon wafers with a uniform layer of photoresist. The pattern to be etched in the silicon was printed on a metal mask which acted like a photographic negative. By a process similar to contact printing, light was shone through the metal mask and those areas of the photoresist exposed to light were depolymerised. Using a mixture of suitable solvents, these depolymerised regions were selectively removed from the silicon wafer. Ion beam etching was then used to etch exposed regions of the silicon wafer. The desired depth was precisely controlled by the etching time. Finally, once the required depth had been reached, the remaining resist material was removed with further suitable solvents.

2.5.2. Preparation of polystyrene replicas

Pieces of silicon wafer bearing known patterns of precise dimensions were placed pattern-side uppermost on glass microscope slides. Pieces of Petri dish were cut to a size approximately 3 mm smaller than the shape of the silicon wafer. These were then cleaned with absolute ethanol to remove grease and placed gently on the wafers, and a glass microscope slide placed on top. At least 18 replicas were usually made simultaneously in any one tray. Ceramic tiles (11 cm square) were placed on top of the slide-plastic-wafer sandwiches in 16 cm x 38 cm trays and approximately 200 g weights applied to give an even downward pressure. These were then placed in an oven pre-heated to 200°C. After at least 40 min, but no longer than 1 h, the trays were removed, taking care to keep them level, and cold water immediately poured into the tray. The replicas and silicon wafers were teased apart if necessary after the rapid cooling. The microfabricated topographies that were used in this study are described in chapter 7, section 2.

2.5.3. Inoculation and incubation of spores on polystyrene replicas

Replicas arranged on glass microscope slides sitting on the surface of 0.4% TWA were inoculated with approximately 3 mg of fresh urediniospores in a settling

tower 23 cm diameter and 76 cm high. After allowing 3 min for the spores to settle, the lids of the Petri dishes were coated with a fine spray of tap water before replacement, and the dishes sealed with parafilm to ensure the retention of 100% RH. The dishes were then kept for 48 h in conditions optimal for spore germination and germ tube growth (i.e. for *Puccinia hordei,* dark at 15°C for 24 h followed by 24 h at 18°C with a 16 h photoperiod; for *P. graminis* f. sp. *tritici*, dark for 3 h at 18°C then 22°C for the remainder with daily 16 h photoperiods).

2.5.4. Preparation of inoculated replicas for fluorescence microscopy and their assessment

After 48 h the germlings were fixed by the addition of drops of lactophenol (BDH Ltd., Dorset, England) to the agar surface taking care not to contaminate the replicas. The dishes were resealed and left at room temperature for at least 1 h. The replicas on slides were then removed from the Petri dishes and stained *in situ* for 5 min with 3% v/v Uvitex BHT in 20% glycerol. The replicas were removed and washed in 20% glycerol before mounting in fresh 20% glycerol on clean microscope slides. Material prepared this way was kept in glass Petri dishes in the dark at 4°C until assessment. The following were scored on at least ten quadrats taken from at least three separate replicas for each treatment: germlings without appressoria but in contact with ridges; germlings with differentiated appressoria; and germlings with appressoria and subsequent infection structures (i.e. vesicles and primary infection hyphae). 1000 and 1800 spores were analysed in each treatment. The numbers of differentiated germlings were expressed as percentages of the total number of germlings.

3. DEVELOPMENTAL MORPHOLOGY OF *PUCCINIA STRIIFORMIS* F. SP. *HORDEI*

3.1. Introduction

This chapter provides a detailed analysis of the developmental morphology of *P. striiformis* f. sp. *hordei* during infection of a susceptible barley cultivar. The infection process, involving stomatal penetration, invasion through the leaf and finally uredinium formation, is described using the correlative techniques of fluorescence microscopy of cleared leaves and LTSEM of frozen-hydrated material. Fluorescence microscopy of cleared, infected leaves stained with fluorochromes which have a high affinity for fungal cell walls, is regarded as an extremely useful and routine method for analysing fungal growth and differentiation within infected host tissue (e.g. see: Rohringer *et al.*, 1977; Niks & Kuiper, 1983; Luke, Barnett & Pfahler, 1984; Scholes & Farrar, 1987; Southerton & Deverall, 1989; Jacobs, 1990; Niks, 1990). LTSEM is now regarded as the method of choice for examining most biological samples for scanning electron microscopy (Jeffree & Read, 1991; Read & Jeffree, 1991) and is particularly suited to studying plant-pathogen interactions (Read, 1991). This chapter and chapters 4, 5 and 6, provide a comparative analysis of the developmental and infection strategies of the markedly contrasting barley leaf rusts *P. striiformis* f. sp. *hordei* and *P. hordei*.

3.2. Results

3.2.1. Pre-penetration phase

Urediniospores which germinated on the leaf surface produced mostly unbranched germ tubes exhibiting directional growth at right angles to the anticlinal cell walls of epidermal cells (Fig. 3). Many germ tubes grew over stomata (not shown), but when penetration did occur, this was without the formation of a morphologically distinct appressorium (Fig. 4). An extracellular secretion was produced on the underside of germ tubes (Fig. 4).

3.2.2. Invasive growth phase

Each germ tube which penetrated a stoma produced a globose, aseptate vesicle directly beneath the guard cells (Figs. 5-7, 9-13). A prominent infection peg was

Figs. 3-8. *P. striiformis* f. sp. *hordei*/barley leaf
Scanning electron micrographs of early stages of infection

Fig. 3. Sporeling growing at right angles to the long axis of the leaf before penetrating a stoma (arrow). 1 dpi. Partially freeze-dried. Bar = 100 μm.

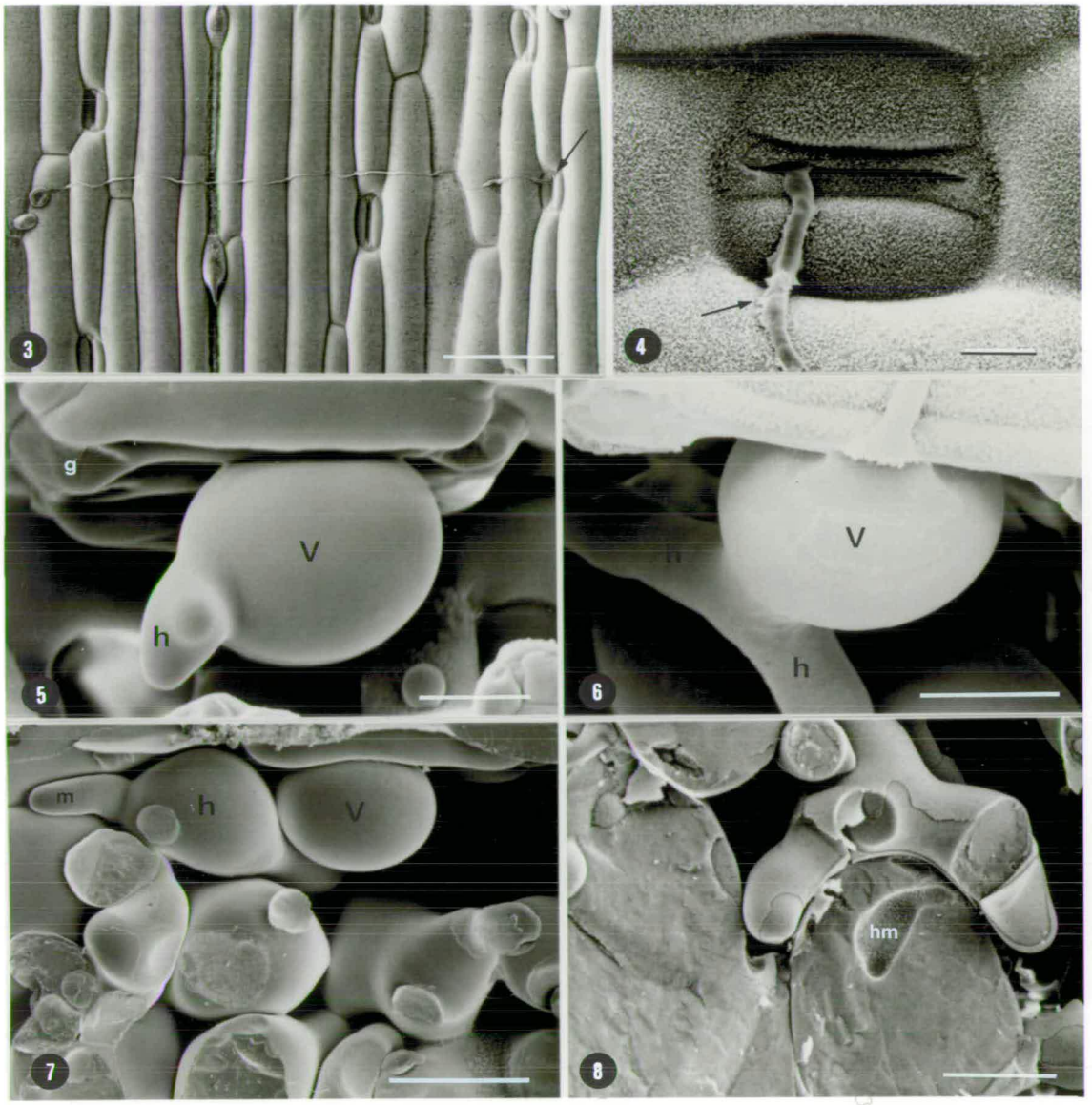
Fig. 4. Penetration through a closed stoma without the formation of a morphologically distinct appressorium. Note the presence of an extracellular secretion on the underside of the germ tube (arrow). 3 dpi. Partially freeze-dried. Bar = 10 μm.

Fig. 5. Fracture through a substomatal cavity. A primary infection hypha (h) has developed from a globose aseptate vesicle (v) positioned directly beneath the guard cells (g) of a stoma. 1 dpi. Fully frozen-hydrated. Bar = 10 μm.

Fig. 6. Top view of a longitudinal fracture through a substomatal cavity. Two aseptate primary infection hyphae (h) have developed from the same place on the vesicle (v) and have grown out into the substomatal cavity. Note the absence of a prominent infection peg. 1 dpi. Fully frozen-hydrated. Bar = 10 μm.

Fig. 7. Fracture through a substomatal cavity showing the vesicle (v) and the swollen primary infection hypha (h) behind the haustorial mother cell (m). 4 dpi. Fully frozen-hydrated. Bar = 20 μm.

Fig. 8. Fracture through a mesophyll cell, a branch of a runner hypha and a haustorial mother cell. The impression of a haustorium (hm) can be seen in the mesophyll cell. Fully frozen-hydrated. 15 dpi. Bar = 20 μm.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Figs. 9-13. *P. striiformis* f. sp. *hordei*/barley leaf
Fluorescence micrographs of early stages of infection

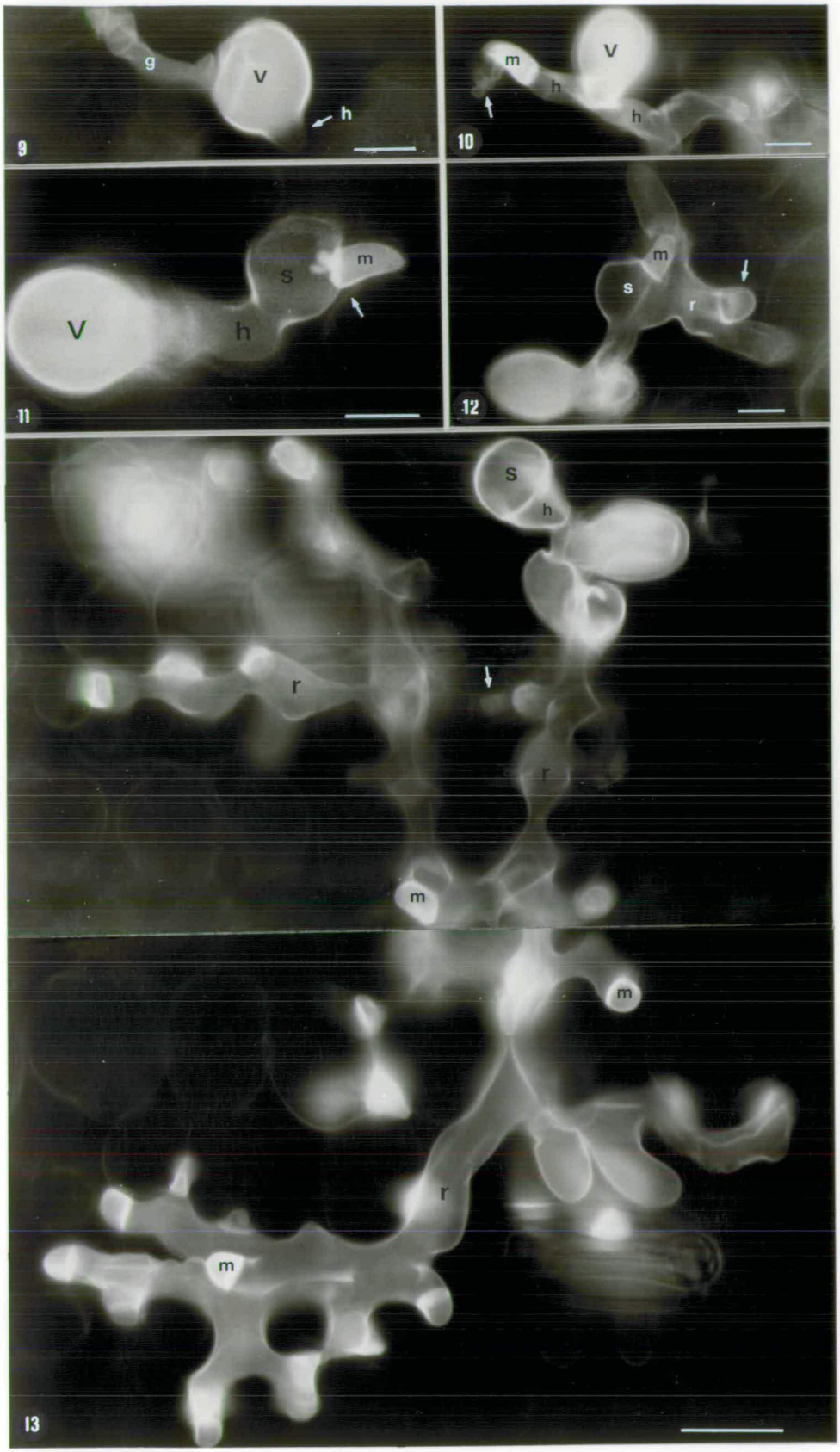
Fig. 9. Primary infection hypha (h) developing from a globose aseptate vesicle (v) within the substomatal cavity. Note the intense staining of the vesicle and the weak staining of the germ tube (g) which in this case has been pulled into the substomatal cavity. 1 dpi. Bar = 5 μm.

Fig. 10. A vesicle (v), with two primary infection hyphae (h). Note that one of these hyphae has an unlobed primary haustorial mother cell (m) and lobed primary haustorium (arrow). The vesicle and haustorial mother cell are more intensely stained than the infection hyphae. 6 dpi. Bar = 5 μm.

Fig. 11. Vesicle (v) and primary infection hypha (h) with swelling (s) behind the haustorial mother cell (m). Note some necrosis (arrow) around the mesophyll cell. 6 dpi. Bar = 5 μm.

Fig. 12. Development of aseptate invasive runner hyphae (r) from the swelling (s) behind the haustorial mother cell (m). Note the development of another haustorial mother cell (arrow) on one of the runner hyphae. 5 dpi. Bar = 5 μm.

Fig. 13. Large aseptate runner hyphae (r) with haustorial mother cells (m) branching beneath the epidermis and down into the mesophyll layer. Note: the swelling (s) in the primary infection hypha (h) and the haustorium (arrow). 6 dpi. Bar = 10 μm.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not evident (Fig. 6). One to three, typically two, large aseptate primary infection hyphae emerged from the same location on the vesicle surface and grew into the substomatal cavity (Figs. 5, 6, 9-13). Occasionally, vesicles and associated primary infection hyphae differentiated on the leaf surface (not shown). This tended to occur where large numbers of germ tubes were in close proximity to each other. Sometimes, however, just a solitary germ tube would develop a vesicle over a stoma. Where vesicles and primary infection hyphae had developed on the leaf surface, a septum delimiting the vesicle could be clearly seen by fluorescence microscopy (not shown). Within the substomatal cavity, an unlobed brightly fluorescing haustorial mother cell, delimited by a septum, differentiated at the tip of a primary infection hypha in contact with a mesophyll cell (Figs. 7, 10-12). In infected leaves after 6 dpi, it was common to find individual aborted attempts at infection where primary infection hyphae, but not haustorial mother cells, had been formed. Within the mesophyll cell, a typically lobed haustorium developed after penetration from the haustorial mother cell (Figs. 8, 10). After the haustorial mother cell and haustorium had formed, a swelling in the primary infection hypha often developed adjacent to the haustorial mother cell (Figs. 7, 11, 12). Overall, vesicles and haustorial mother cells stained more prominently with Uvitex than did germ tubes, primary infection hyphae or haustoria (Figs. 9-12). Fungal growth did not advance beyond the substomatal cavity until after 4 dpi during which time the vesicle and the primary infection hyphae adjacent to the swelling perceptibly increased in diameter.

Large (8.0 - 13.3 μm wide) aseptate *runner hyphae* formed as branches from primary infection hyphae (Figs. 12, 13) and rapidly grew between cells within the subepidermal and mesophyll layers (Figs. 13, 14, 17). In the context of this thesis, runner hyphae are defined as large, rapidly growing, invasive hyphae that grow for long distances within the leaf. Haustorial mother cells and lobed, often bifurcate, haustoria were associated with almost all mesophyll cells encountered by these hyphae (Figs. 13, 14, 17). The aseptate runner hyphae grew between and around leaf veins to produce a diffuse colony in which much of the available intercellular air spaces were not colonized (Fig. 17).

3.2.3. Reproductive phase

Nine to ten dpi there was increased hyphal branching in areas of the colony some distance from the point of penetration and this activity was indicative of sites where uredinia subsequently developed (Figs. 19, 23). The hyphal branching was accompanied by the formation of thin uredinial bed hyphae as branches from the

Figs. 14-18. *P. striiformis* f. sp. *hordei*/barley leaf
Fluorescence micrographs of invasive hyphae and haustoria

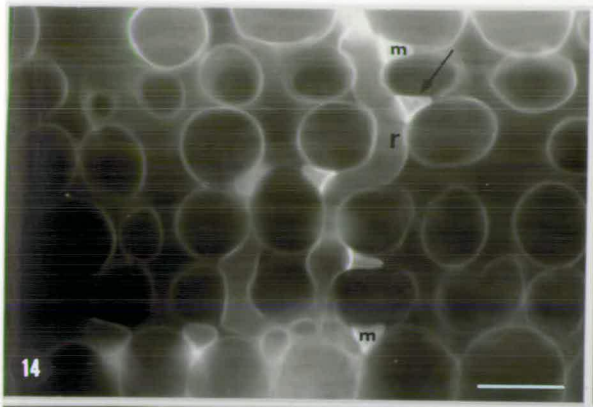
Fig. 14. Aseptate runner hypha (r) with haustorial mother cells (m) at mesophyll cell junctions. Note the pore (arrow) in the haustorial mother cell. 9 dpi. Bar = 10 μm.

Fig. 15. Mesophyll cell containing a pear-shaped haustorium (arrow) derived from a branch of a septate runner hypha. 19 dpi. Bar = 10 μm.

Fig. 16. Septate runner hypha (r) growing away from a uredinial bed (b) and producing pear-shaped haustoria (arrows) within mesophyll cells. 15 dpi. Bar = 10 μm.

Fig. 17. Branches of aseptate runner hyphae with haustorial mother cells giving a diffuse colony. Note the vesicle at the penetration site (v) and the greater concentration of hyphae along leaf veins (asterisks). 10 dpi. Bar = 50 μm.

Fig. 18. Edge of a mature uredinium (u). Hyphae around the mesophyll cells originate from the septate runner hypha (r) running along the edge. Note the presence of pigment (arrows) in the uredinial bed. 15 dpi. Bar = 5 μm.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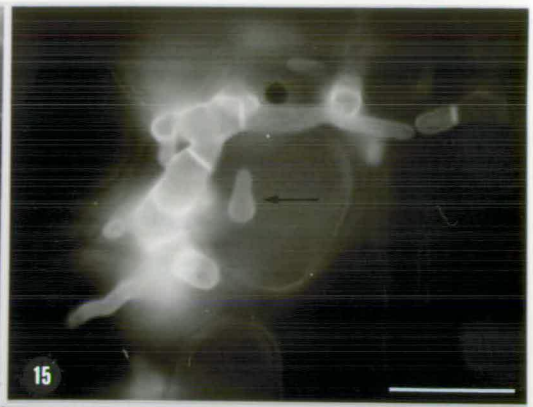
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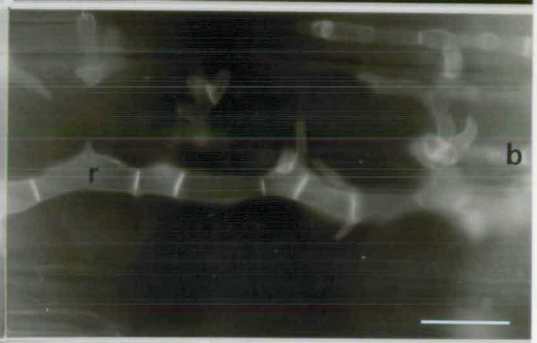
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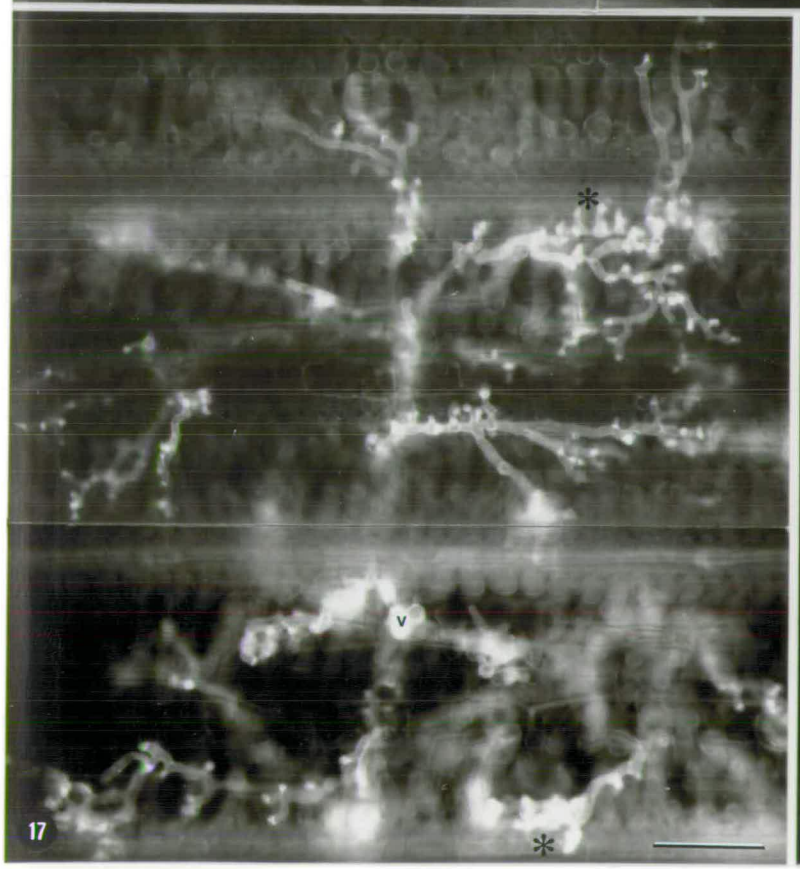
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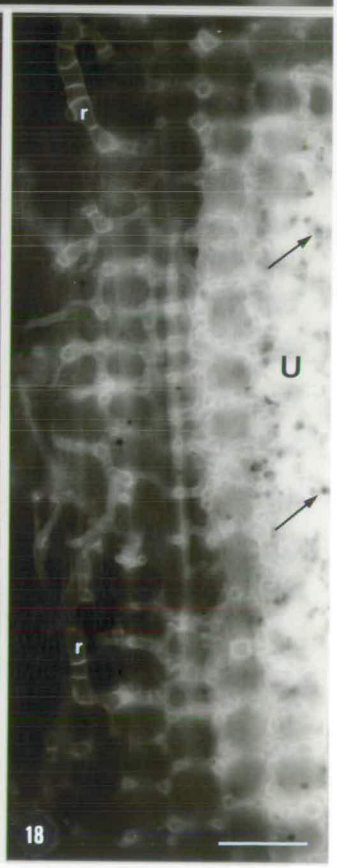
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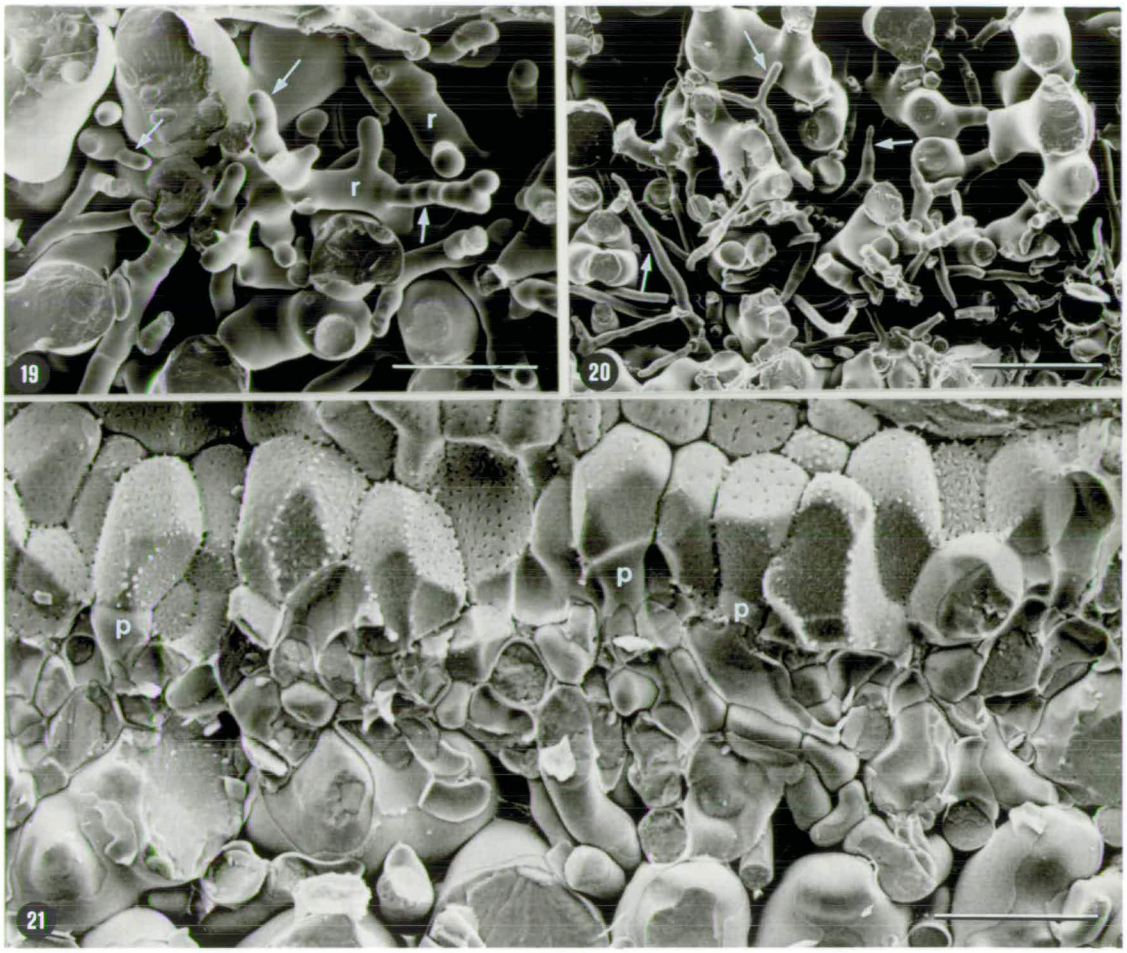
Figs. 19-21. *P. striiformis* f. sp. *hordei*/barley leaf
Scanning electron micrographs of stages in uredinial bed formation

Fig. 19. Fracture showing runner hyphae (r) and branches of thin septate uredinial bed hyphae (arrows) at an early stage in the formation of a primary uredinial bed. 9 dpi. Fully frozen-hydrated. Bar = 30 μm.

Fig. 20. Fracture showing the thin, spiky, uredinial bed hyphae (arrows) at an early stage in the formation of a secondary uredinial bed. 15 dpi. Fully frozen-hydrated. Bar = 60 μm.

Fig. 21. Fracture through a uredinial bed showing septate uredinial bed hyphae and developing urediniospores with a distorted morphology. The urediniospores are borne on short pedicels (p). Note the absence of paraphyses. 15 dpi. Fully frozen-hydrated. Bar = 20 μm.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Figs. 22-28. *P. striiformis* f. sp. *hordei*/barley leaf
Fluorescence micrographs of the reproductive phase

Fig. 22. Production of a thin uredinial bed hypha (arrow) of the type which gives rise to the primary uredinial bed. Note the apical haustorial mother cells (m) on the short branches. 10 dpi. Bar = 20 μm.

Fig. 23. An early stage in the formation of the primary uredinial bed by the profusely branching uredinial bed hyphae. Note the initiation of septation (arrow) in fungal tissue around the mesophyll cells (cf. the later stage shown in Fig. 24). 10 dpi. Bar = 20 μm.

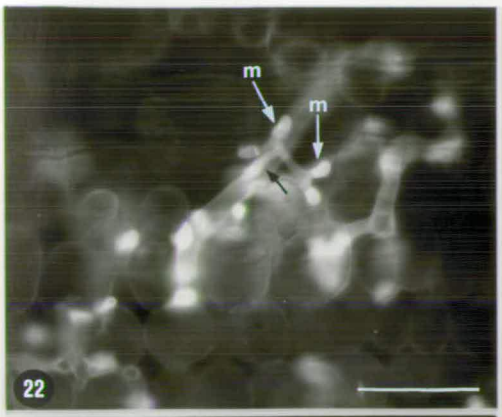
Fig. 24. Development of the multi-septate, subepidermal layer of the uredinial bed by cohesion and profuse septation of hyphae originating as branches of runner hyphae around mesophyll cell junctions. 15 dpi. Bar = 10 μm.

Fig. 25. Septation progressing in the runner hypha (r) back from the primary uredinial bed (b) toward the original site of penetration. 10 dpi. Bar = 20 μm.

Fig. 26. The development of secondary uredinial beds (b) from the spiky uredinial bed hyphae produced by the septate runner hyphae. 20 dpi. Bar = 50 μm.

Fig. 27. Mature uredinia merging along the leaf length. Note the absence of paraphyses among the pigmented urediniospores in the uredinium and the presence of runner hyphae (r) within the leaf. 15 dpi. Bar = 50 μm.

Fig. 28. Uredinial beds at varying stages of development linked by a ramifying network of runner hyphae. The more immature uredinial beds are located at the leading edge of the infection (arrows) towards the base of the leaf. 12 dpi. Bar = 120 μm.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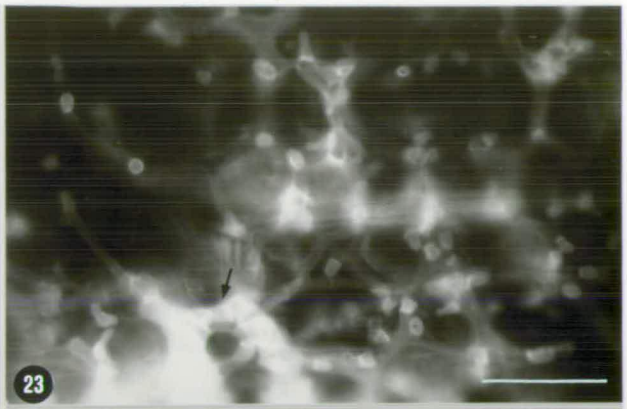
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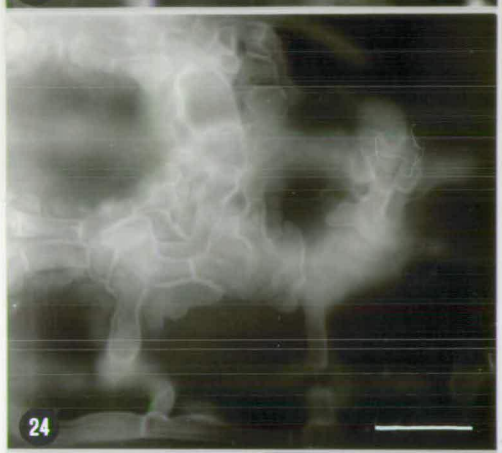
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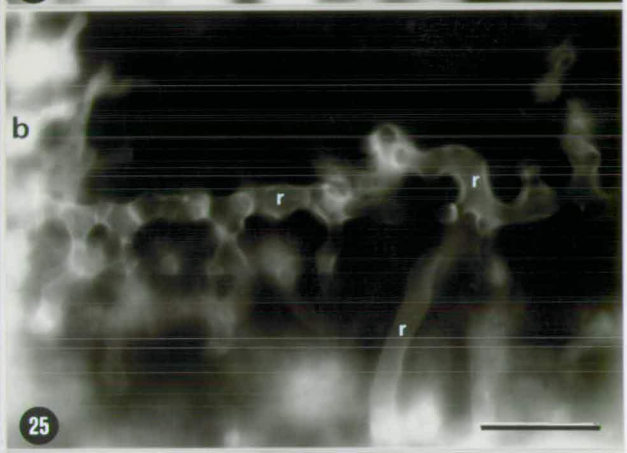
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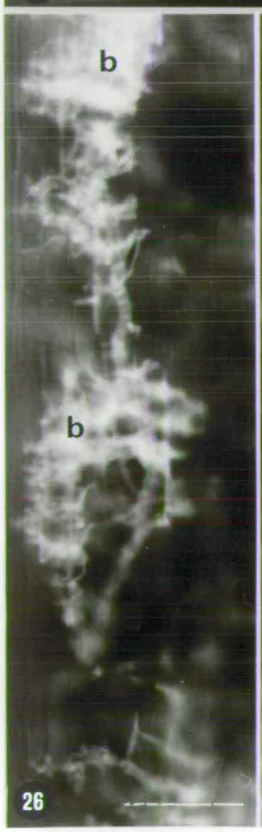
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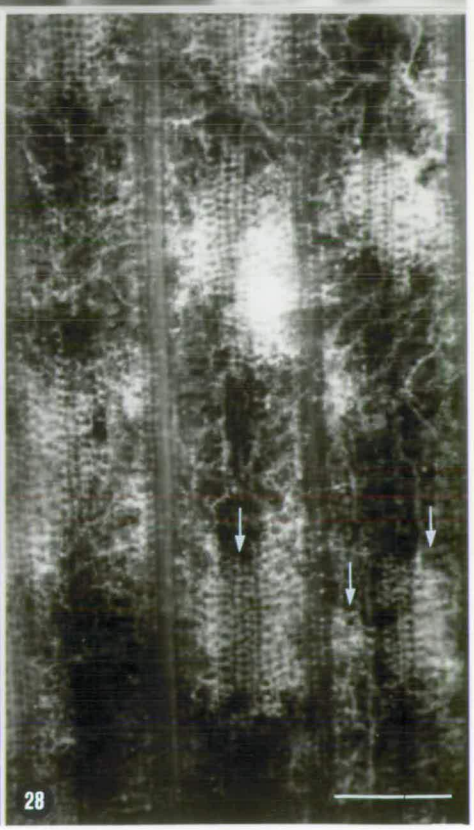
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aseptate runner hyphae. The uredinial bed hyphae rapidly became highly branched and produced abundant terminal haustorial mother cells (Figs. 19, 22). The uredinial bed hyphae and the aseptate runner hyphae ramified around mesophyll cells and at the same time underwent considerable septation (Fig. 23). Some of these hyphal compartments contained pigment (Fig. 18) which was brown in colour (not shown) when observed by brightfield light microscopy. Further growth and development of the uredinial bed resulted in a coherent mass of subepidermal tissue which separated the epidermis from the mesophyll and subsequently gave rise to urediniospores (Figs. 21, 24).

Once septation began in the uredinial bed, it also started in the initially aseptate runner hyphae and steadily moved back in a wave along these hyphae towards the vesicle at the primary site of infection (Fig. 25). However, the large runner hyphae growing in the opposite direction (i.e. from the uredinial bed towards the young colony margin) remained aseptate at this time (not shown) but subsequently became septate as the uredinium matured. These multi-septate runner hyphae grew relatively straight, mostly in the subepidermal layer and mesophyll under the adaxial leaf surface. They grew down the length of the leaf and developed branches, haustorial mother cells and lobed or pear-shaped, haustoria along their lengths (Figs. 8, 15, 16). These runner hyphae also produced branches which had a similar appearance to their parent hyphae and which grew across leaf veins (Fig. 28).

Secondary uredinia developed at intervals along the leaf, mainly on the adaxial surface, from concentrations of thin uredinial bed hyphae formed as branches from the large, septate runner hyphae. These uredinial bed hyphae initially lacked haustorial mother cells and had a spiky appearance (Figs. 16, 18, 20, 26). They formed a ramifying network of coherent tissue, along with associated runner hyphae, around adjacent mesophyll cells (Figs. 18, 24, 28) in much the same way that uredinial bed hyphae formed the uredinial bed of a primary uredinium (Fig. 23).

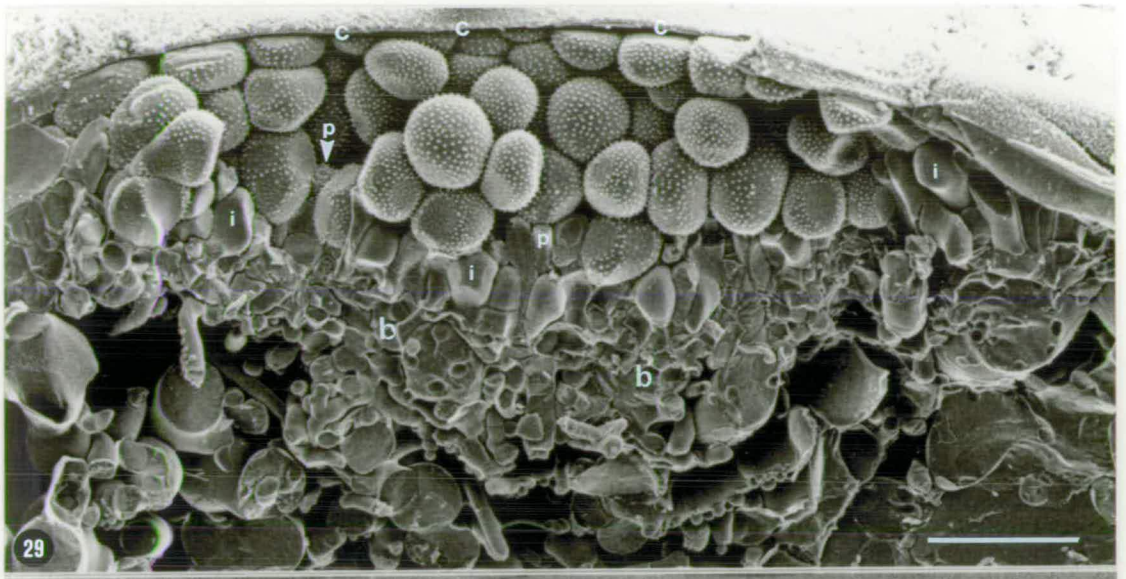
Unbranched hyphae arising from the uredinial bed (*sporogenous cells*) grew out towards the leaf surface, became septate and formed terminal cells which differentiated into urediniospores and subterminal cells which developed into the short supporting pedicels (Figs. 21, 29). All the cells appeared to be embedded in an extracellular matrix (Fig. 21). Urediniospores were continuously produced from the uredinial bed all around the base and periphery of the uredinium with urediniospore development progressively more advanced toward the centre of the uredinium (Figs. 21, 29-31). Continued spore formation caused the cuticle and

Figs. 29-31. *P. striiformis* f. sp. *hordei*/barley leaf
Scanning electron micrographs of secondary uredinia in established infections

Fig. 29. Fracture through a uredinium before emergence through the leaf surface. Note distorted mature urediniospores appressed against the underside of the cuticle (c). A layer of septate fungal tissue around the mesophyll cells constitutes the uredinial bed (b). Short pedicels (p) and urediniospore initials (i) without spines can be seen at the periphery of the uredinium. Note also the absence of sterile tissue around the periphery of the uredinium and the general absence of paraphyses. Fully frozen-hydrated. 15 dpi. Bar = 40 μm.

Fig. 30. Top view of uredinia erupting through the cuticle and epidermis along the anticlinal junctions between epidermal cells. The larger, more mature urediniospores are located towards the centre of the uredinium, and are globose and echinulate. 9 dpi. Partially freeze-dried. Bar = 50 μm.

Fig. 31. High power of the edge of the uredinium in Fig. 30 showing different stages in urediniospore maturation. The uredinium here has erupted at the junction between a companion cell (c) and epidermal cell. The urediniospore initials (i) lack surface spines and are embedded in extracellular matrical material (arrows). As the urediniospores (us) mature they become more globose and echinulate. Fully frozen-hydrated. 9 dpi. Bar = 10 μm.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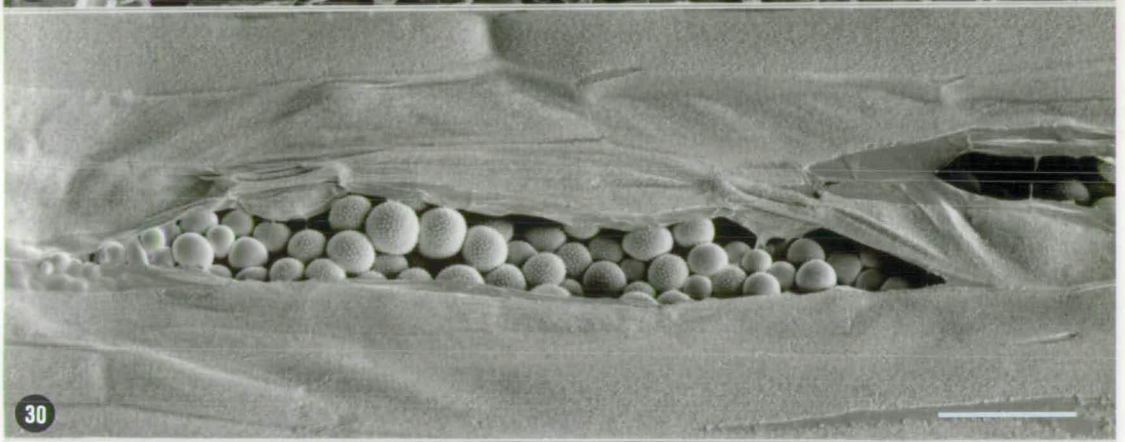
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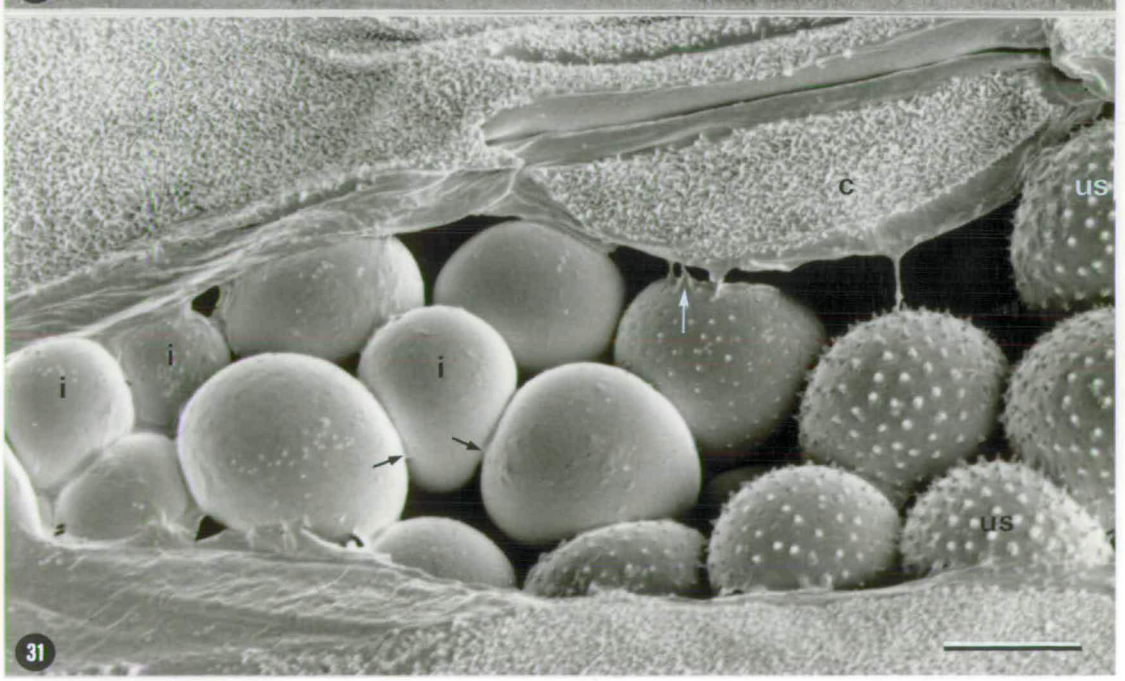
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epidermis to stretch and eventually rupture, usually along the anticlinal junctions between epidermal cells (Figs. 30, 31). Initially the urediniospore initials lacked surface spines (Figs. 21, 29) and this was most evident around the periphery of uredinia which had erupted through the epidermis (Figs. 30, 31). Young spores tended to have a distorted morphology filling all the available space around them (Figs. 21, 29) and appeared to be covered with the extracellular matrical material (Fig. 21). As they matured they became more globose although mature spores closely appressed against the underside of the cuticle were flattened (Fig. 29). The cytoplasm within the spores at the top of the uredinium was pigmented by this stage (Fig. 27). Paraphyses were not obvious, if present at all, within the uredinium (Figs. 21, 27, 29). The opening in the uredinium revealed the presence of strands of extracellular matrical material around each spore (Fig. 31).

After about 11 dpi, the colony took on the appearance of developing uredinia linked by runner hyphae (Figs. 26-28), with the more mature uredinia generally located towards the tip of the leaf. Many uredinia merged (Fig. 27) and, when they broke through the cuticle (Fig. 30), typically produced stripes of sporulation characteristic of the macroscopic symptoms of yellow rust where adjacent interveinal regions were infection-free.

3.3. Discussion

A number of features characterise *P. striiformis* from other cereal rusts, notably: the absence of an appressorium (Pole-Evans, 1907; Allen, 1928); large and aseptate invasive runner hyphae are formed; morphologically distinct hyphae for uredinial bed formation are formed; a delayed latent period and a semi-systemic growth pattern occurs (Allen, 1928); and a sexual phase is apparently absent (Hassebrauk, 1970).

The techniques of fluorescence microscopy and LTSEM have been used correlatively to provide complementary information on some of these features. A more complete picture of the temporal and spatial aspects of fungal development within the host has been obtained than would have been possible if the two techniques had been used in isolation. Fluorescence microscopy can only provide a two dimensional view of structures in the same focal plane but will reveal septation, pigmentation and host cell necrosis. On the other hand, LTSEM gives a more three dimensional perspective of colony development within the leaf. Furthermore, the latter method provides excellent preservation, especially because

water is retained *in situ* and labile components (e.g. extracellular matrices) are not removed by chemical treatments and solvent action (Beckett & Read, 1986; Jeffree & Read, 1991; Read, 1991; Read & Jeffree, 1991).

The germ tubes of *P. striiformis* grew preferentially across the axis of the leaf (Fig. 3) which confirms the observations of Mares & Cousen (1977), Cartwright & Russell (1981) and Opel *et al.* (1986*a*). On graminaceous hosts, the stomata are arranged in parallel staggered rows and directional growth would therefore increase the probability of the germ tube encountering a stoma (Read *et al.*, 1992). The ability of rust germ tubes to exhibit growth orientation has been well documented as a contact-mediated response (Hoch & Staples, 1991; Read *et al.*, 1992; and see section 1.6). Close contact between the germ tube and the substratum is essential for the sensing of surface topography (Epstein *et al.*, 1985) and the extracellular matrix between the underside of the *P. striiformis* germ tube and the leaf (see Fig. 4) may provide an adhesive in this respect.

Although some swelling of germ tube tips over stomata may occur, a morphologically distinct appressorium is not formed either before penetration, or before differentiation of a vesicle and infection hyphae, as sometimes seen on the surface of the leaf (Kellock, L.J. & Lennard, J.H., unpublished). A septum was not seen in the germ tube before entry into the leaf and Allen (1928) suggested that the lack of this septum in *P. striiformis* would contribute to the absence of an appressorium. Septa may be important for physically isolating adjacent cell compartments allowing them to undergo alternative differentiation events (Gull, 1978; Read, 1994) and this may be important during the infection process of *P. striiformis*. In most rusts, appressorium formation is accompanied by a mitotic division of the two nuclei within the germ tube. The resultant four nuclei become compartmentalised within the appressorium by being separated from the germ tube by a septum (e.g. in *P. graminis*, Allen, 1926; *Uromyces phaseoli*, Maheshwari *et al.*, 1967; *U. appendiculatus*, Kwon & Hoch, 1991). In *P. striiformis*, in which neither a septum or a morphologically recognisable appressorium differentiates from a germ tube, it has been shown that the germ tube can contain 4-10 nuclei (Little & Manners, 1969; Goddard, 1976) with usually 2 nuclei at the germ tube tip (Allen, 1928; Little & Manners, 1969). It is not clear whether mitosis occurs on contact with the stomatal complex or not. However, evidence suggesting that signals derived from the stomatal complex are not involved, has been provided by Little & Manners (1969) who have shown that mitosis occurs in germ tubes of *P. striiformis* on agar alone.

The only septa which form in *P. striiformis* before uredinial bed formation are those which delimit germ tubes from vesicles and associated primary infection hyphae (Kellock, L.J. & Lennard, J.H., unpublished), and vesicles from haustorial mother cells. This contrasts with most other rusts in which the intercellular infection hyphae are septate (Harder, 1984) as is typical of basidiomycetes (Littlefield & Heath, 1979). The absence of a general septation in the infection hyphae of *P. striiformis* is therefore exceptional. The presence of aseptate infection hyphae was recorded in some of the earliest developmental studies of *P. striiformis*. For example, Ward (1905) and Marryat (1907) noted that septa developed just before spore formation to delimit binucleate cells in the large, multinucleate and previously aseptate hyphae. Binucleate cells within the infection hyphae of yellow rust were found not to occur until 8-10 dpi (Wright, 1976), corresponding with the delay in general septation observed in the present study. Septa will restrict nuclear migration (Littlefield & Heath, 1979) and, as indicated earlier, promote cell specialization. Septation was thus correlated here with the differentiation of specialized cells (vesicles, haustorial mother cells, cells within the uredinial bed, pedicels and urediniospores). Why *P. striiformis* forms aseptate infection hyphae prior to uredinium formation is not clear, particularly since these hyphae are septate in other rusts (e.g. *P. hordei*, chapter 4). Possible advantages of aseptate infection hyphae might be to: 1) provide a rapid interchange of food materials, 2) facilitate nuclear migration and prevent the formation of dikaryotic cell compartments thus permitting possible genetic re-assortment between fused colonies (see section 6.3.7), 3) prevent cell specialization, and/or 4) facilitate an increased growth rate because energy and materials involved in septum formation are not involved. The latter is supported by the finding that, before uredinium formation, *P. striiformis* spreads faster than *P. hordei* within the leaf (see sections 5.2.2 and 5.3.2). Aseptate hyphae tend to have powers of rapid extension, which allow for the rapid utilisation of food resources (Cooke & Rayner, 1984). In certain species of *Phlebia*, basidiomycetes which cause wood-decay, the peripheral zone of colonies grown in culture is composed of sparsely branched, rapidly extending, aseptate hyphae. Behind this front is a system of highly branched, septate hyphae which, unlike the aseptate hyphae, are capable of hyphal fusions and antagonizing hyphae of other fungi (Cooke & Rayner, 1984). This pattern of colony development is analogous to that of *P. striiformis* where general septation only occurs after a prolonged period of exploratory growth. The diffuse nature of the *P. striiformis* colony, prior to the formation of the primary uredinium, may be

a consequence of the lack of a general septation; septate intercellular hyphae of other rusts branch profusely (e.g. *P. hordei*, chapter 4).

Being the first cell differentiated from the germ tube, the vesicle of *P. striiformis* may possess some characters in common with the appressoria of other rusts. The septum formed between the germ tube and the vesicle may be of the 'appressorial-type', namely non-perforated (Littlefield & Heath, 1979). This may allow food reserves to accumulate in the vesicle during the lag phase to initially 'fuel' the infection hyphae before haustoria, formed from haustorial mother cells, can absorb nutrients from host cells. After the lag phase and up to uredinial bed formation, the diffuse but extensive spread of the large and aseptate runner hyphae, accompanied by the formation of haustorial mother cells and associated haustoria, may represent a vigorous system for transporting nutrients to the reproductive centres (see above). Septation may then serve to compartmentalise and concentrate these accumulated nutrients along with nutrients supplied by the uredinial bed hyphae. Finally, septation in the runner hyphae, initially developed back from primary uredinial beds to the site of penetration, may prevent the dissipation of this 'nutrient-sink' from the areas of reproduction. The role of these aseptate runner hyphae as a nutrient transport system to areas of reproduction, is supported by TEM observations made by Mares (1979) on the growth of *P. striiformis* in wheat leaves. In Mares' study, it was suggested that metabolites were stored in a non-diffusible form in the aseptate hyphae until required for reproduction. As the uredinia developed, storage deposits in the septate hyphae adjacent to developing uredinia disappeared.

P. striiformis produces morphologically distinct hyphae for uredinial bed formation. These uredinial bed hyphae are thinner than runner hyphae and are capable of encircling mesophyll cells. In primary uredinial beds, the uredinial bed hyphae are very short and possess terminal haustorial mother cells. The appearance of these hyphae, which were described by Allen (1928) as 'feeding hyphae', is the first recognizable sign of uredinia. Similarly, the development of the spiky hyphae on septate runner hyphae is always indicative of the initiation of a secondary uredinium. The repeated septation of branches of runner hyphae and uredinial bed hyphae gives a subepidermal layer of sporogenous tissue embedded in an extracellular matrix. It is not known whether the matrix is of host or fungal origin (Beckett & Woods, 1987) but the presence of material of similar appearance around maturing spores near the top of the *P. striiformis* uredinium (Fig. 31) suggests a fungal origin. A mucilaginous layer on the surface of yellow rust spores which apparently changes in appearance with different conditions of humidity has

also been reported (Stanbridge & Gay, 1969; Rapilly, 1979). In addition, adhesion of yellow rust spores to the substratum is far greater in high relative humidities and on smooth rather than rough surfaces (Rapilly & Foucault, 1976). Mucilagenous material around urediniospores may permit good retention of spores on the leaf surface during the conditions of high humidity which initiate germination (Beckett *et al.*, 1990). In *Uromyces viciae-fabae*, the retention of living urediniospores on the leaf surface is achieved by the formation of an adhesion pad (Deising *et al.*, 1992). On synthetic surfaces, the extracellular matrix responsible has been shown to be released from urediniospores over three stages, from imbibition through to germ tube growth, and during this time both passive and active phases of adhesion are involved (Clement *et al.*, 1993*a,b*).

Urediniospores and pedicels of *P. striiformis* f. sp. *hordei* seem to follow a similar course of development to those of other rusts (e.g. *P. coronata* and *P. graminis,* Harder, 1976; *Uromyces viciae-fabae*, Beckett & Porter, 1982, Beckett & Woods, 1987). The sporogenous cells form from the coherent mass of fungal tissue of the uredinial bed and extend apically to form pedicels and spores. However, the pedicels arising from the sporogenous cells are very short unlike those in the uredinia of other rust fungi (e.g. *U. viciae-fabae*, Beckett & Porter, 1982). Paraphyses were not observed. The structures described as paraphyses by Opel *et al.*, (1986*b*) in their SEM preparations resemble the immature spores on pedicels observed in my samples prepared for LTSEM. Some brightly fluorescing cells within uredinia in some of my fluorescence micrographs might be confused for paraphyses (Fig. 27; see section 4.3). However, I interpret these to be immature sporogenous cells. Around the edges of uredinia there was no evidence of sterile tissue: the uredinia were surrounded by sporogenous-type tissue or immature spores. The short pedicels and the absence of paraphyses could account for the relatively flat profile of the *P. striiformis* uredinium. The absence of any sterile boundary tissue may enhance the ease with which uredinia frequently merge to give long stripes of sporulation.

4. DEVELOPMENTAL MORPHOLOGY OF *PUCCINIA HORDEI*

4.1. Introduction

This chapter contains a detailed analysis of the developmental morphology of *P. hordei* during infection of the susceptible barley cultivar Golden Promise. As in the last chapter, the correlative techniques of fluorescence microscopy and LTSEM have been employed.

4.2. Results

4.2.1. Pre-penetration phase

Within the first 24 h after inoculation, urediniospores germinated and produced branched primary germ tubes which typically grew perpendicular to epidermal cell wall junctions which they encountered (Fig. 32; Fig. 65 in chapter 7). Directional germ tube growth with this orientation also occurred on plastic replicas of microfabricated wafers possessing closely spaced ridges (Fig. 64 in chapter 7). The germ tubes usually formed short lateral side branches over the cell junctions (Fig. 32; Fig. 65 in chapter 7). Sometimes, however, these branches produced further branches and these lateral branches were not always developed over epidermal cell junctions (Fig. 32).

Penetration of the leaf occurred through the stomatal pore after the formation of a terminal, morphologically distinct appressorium either on a germ tube branch or, more typically, on the primary germ tube itself (Figs. 32, 36). The appressorium was elongated in shape covering most of the stoma and typically had small lobes at one or more of its 'corners' (Figs. 32, 36; Fig. 65 in chapter 7). The urediniospore and germ tube often collapsed after the formation of the appressorium which in turn collapsed after penetration (Figs. 32, 49; Figs. 64, 65 in chapter 7).

4.2.2. Invasive growth phase

Once within the substomatal cavity, an elongated vesicle, usually orientated along the long axis of the leaf, developed below the guard cells (Figs. 33, 37, 38, 40). A brightly fluorescing septum formed between the penetration peg and the vesicle and in the case of multiple penetrations, which was a common occurrence, vesicles became detached at this septum (Fig. 38).

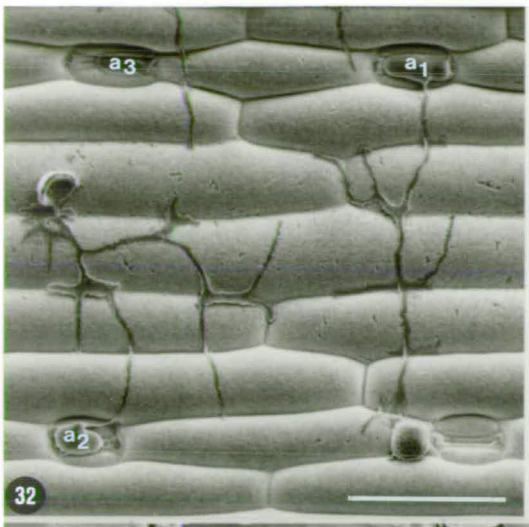
Figs. 32-35. *P. hordei*/barley leaf
Scanning electron micrographs of early stages of infection (< 9 dpi)

Fig. 32. Germinated urediniospores with branched germ tubes growing across epidermal cell wall junctions, over which short lateral side branches typically develop. Morphologically distinct appressoria on the primary germ tube (a_1, a_2), and on a side branch (a_3) have developed over stomatal complexes. Note that the germ tubes have collapsed because their contents have moved into the appressoria. One of the appressoria (a_3) has also collapsed because its cell contents have subsequently migrated into the vesicle within the substomatal cavity. 22 hpi. Partially freeze-dried. Bar = 80 μm.

Fig. 33. Fracture through a penetration site showing the vesicle (v) orientated along the long axis of the leaf with primary infection hyphae (h). Note the septa (s) in the centre of the vesicle and in the primary infection hyphae. Fully frozen-hydrated. 18 hpi. Bar = 20 μm.

Fig. 34. Fracture through a colony showing branching septate hyphae around mesophyll cells. Note the edge of the primary uredinium (u). Fully frozen-hydrated. 9 dpi. Bar = 40 μm.

Fig. 35. High power of the colony in Fig. 34 showing a fractured haustorium (hm) within a mesophyll cell encircled by hyphae. Fully frozen-hydrated. 9 dpi. Bar = 10 μm.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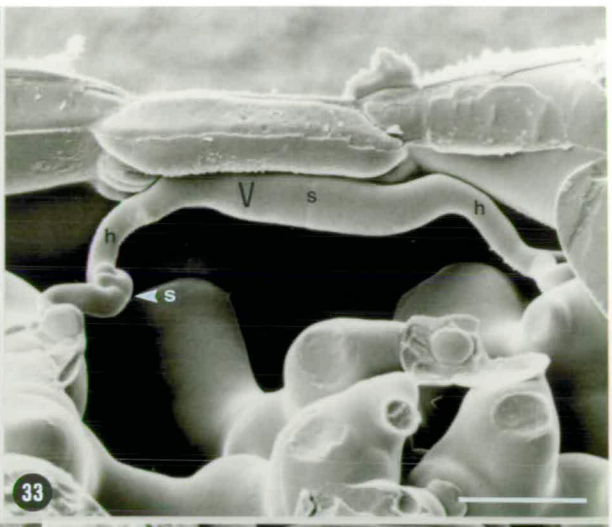
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Figs. 36-43. *P. hordei*/barley leaf
Fluorescence micrographs of early stages of infection (< 7 dpi)

Fig. 36. Urediniospore (us), germ tube (g) and lobed appressorium (a). Note the poor staining of the urediniospore and that the appressorium stains more intensely than the germ tube. 1 dpi. Bar = 10 μm.

Fig. 37. Lower focal plane through the penetration site imaged in Fig. 36 showing the septate vesicle (v) orientated along the long axis of the stoma. Primary infection hyphae (h) with terminal haustorial mother cells (m) have developed at each end of the vesicle. Septation has occurred between the vesicle and primary infection hyphae and between the primary infection hyphae and haustorial mother cells. Note the bifurcate haustorial mother cell (m_1) and development of a secondary infection hypha (sh). 1 dpi. Bar = 10 μm.

Fig. 38. Vesicle (v) with primary infection hyphae (h) and haustorial mother cells (m) detached at the septum (arrow) between the penetration peg and vesicle. 1 dpi. Bar = 10 μm.

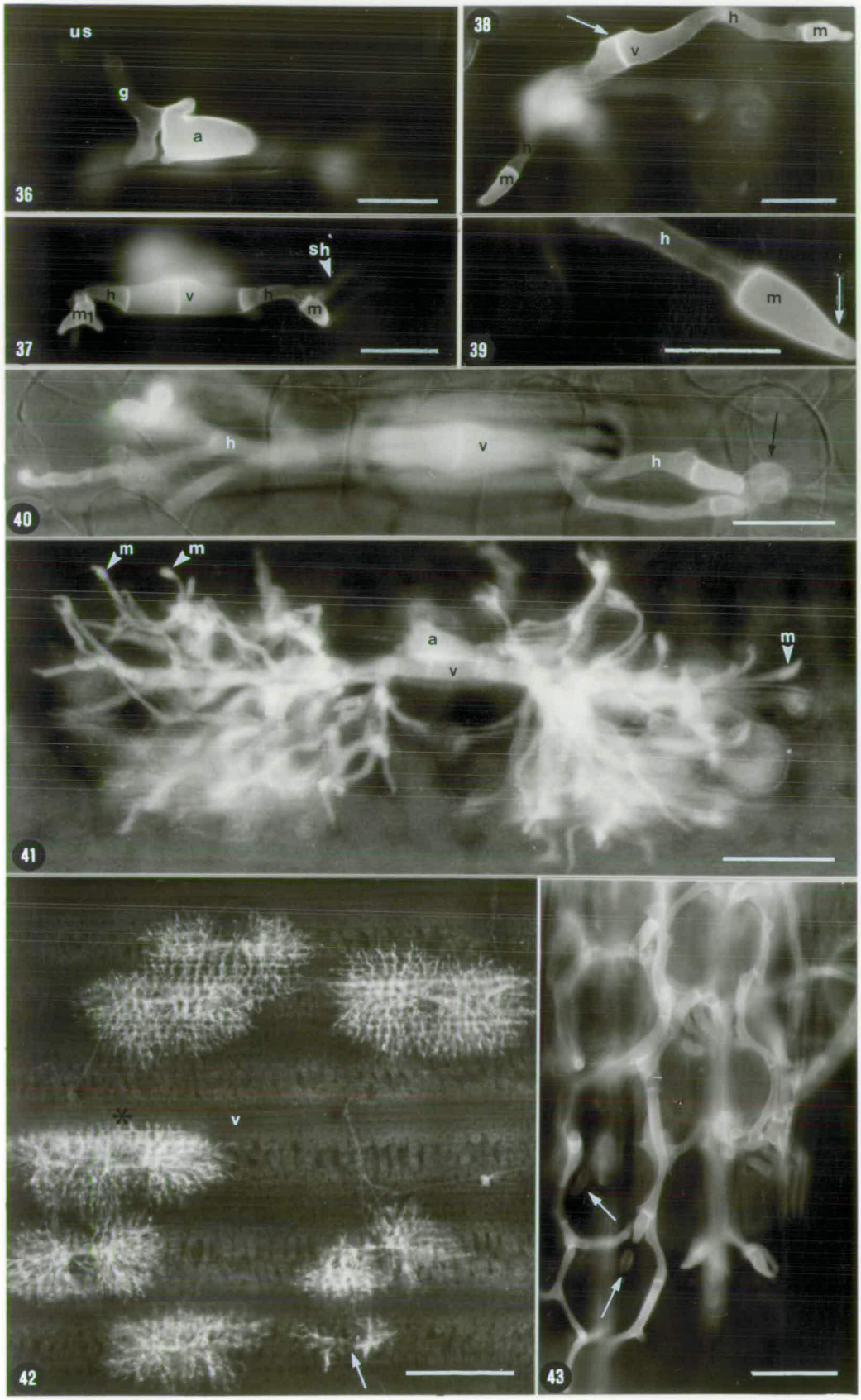
Fig. 39. Primary infection hypha (h) with a terminal haustorial mother cell (m) in which the pore (arrow) at the site of penetration of the mesophyll cell can be seen. 1 dpi. Bar = 5 μm.

Fig. 40. Combined fluorescence and bright field light micrograph. Note that the secondary infection hyphae commonly arise behind septa of the primary infection hyphae (h). The primary infection hyphae are formed at each end of the vesicle (v). Note the haustoria (arrow) within mesophyll cells. 2 dpi. Bar = 10 μm.

Fig. 41. Characteristic 'bow tie'-shaped colony resulting from secondary hyphal formation at each end of the vesicle (v). Note: the original appressorium (a) located above the vesicle; the haustorial mother cells (m). 3 dpi. Bar = 20 μm.

Fig. 42. Individual colonies within a leaf. Each resulted from a single penetration event. Note: a major leaf vein (v) has inhibited the lateral development of one of the colonies (asterisk); the delayed growth of one of the colonies (arrow). 4 dpi. Bar = 100 μm.

Fig. 43. Edge of a colony showing hyphae developing a network around mesophyll cells in which haustoria (arrows) are produced. 7 dpi. Bar = 10 μm.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One primary infection hypha typically emerged at each end of the bipolar vesicle and each terminated in a haustorial mother cell (Fig. 33) which stained strongly with Uvitex (Figs. 37-40). The haustorial mother cells were generally elongated in shape and tapered at the end (Figs. 38, 39), and were sometimes bifurcate (Fig. 37). A pore in the haustorial mother cell (Fig. 39) represented the site of penetration into the mesophyll cell where a bifurcate or ellipsoid haustorium subsequently developed (Figs. 35, 40, 43). The formation of the haustorial mother cells was followed by septation in the middle of the vesicle (Figs. 33, 38) and subsequently at either end of the vesicle (Fig. 37). New, and slightly smaller (2.7 - 4.5 μm wide), septate hyphae branched either from behind the septa at the end of the substomatal vesicle or from behind the septa delimiting haustorial mother cells from the primary infection hyphae (Figs. 37, 40 and 41). By 2 dpi, more hyphal branching had occurred from behind septa associated with the vesicle and the primary infection hyphae (Figs. 40, 41).

Resistance reactions, characterised by necrosis, commonly occurred during these early stages of infection. Growth would often continue at only one end of the vesicle if necrosis was present at the other end (Fig. 42). Otherwise, by 3 dpi, many new septate hyphae had developed in the region near the ends of the vesicle, and repeated hyphal branching gave the colony a characteristic 'bow tie' appearance (Fig. 41). The hyphae, some with terminal haustorial mother cells, grew both between the mesophyll and epidermal layers and down into the mesophyll layer (Figs. 34, 41).

By 4 dpi the growth pattern of the colony changed. Starting at the centre of the colony, the hyphae formed a dense network of interwoven hyphae around mesophyll cells (Fig. 43) in the ellipsoidal colony (Fig. 42). Haustoria were formed within the encircled mesophyll cells (Figs. 34, 35, 43), but at the colony edge haustorial mother cells and haustoria were often formed from hyphae which grew for some distance without branching (not shown). At this time, many colonies in close proximity began to merge but were frequently still unable to grow over the major leaf veins and instead became more densely packed (Fig. 42), possibly as a result of the closer spatial arrangement of these lignified plant cells.

4.2.3. Reproductive phase

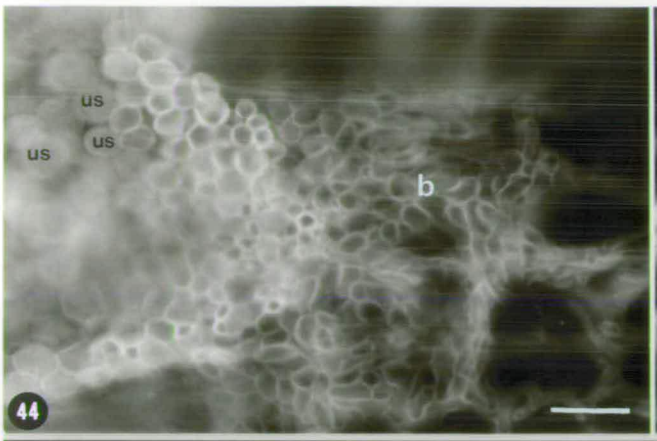
Between 4-5 dpi the hyphae around the mesophyll cells in the centre of the colony (i.e. around the site of penetration), became markedly septate leading to the formation of a subepidermal, coherent, multi-septate layer of fungal tissue (Fig. 44) with pigmentation evident in some of the cell compartments (not shown). This

Figs. 44-46. *P. hordei*/barley leaf
Fluorescence micrographs of the reproductive phase

Fig. 44. Edge of a maturing uredinium showing the subepidermal, multi-septate layer of coherent fungal tissue which constitutes the uredinial bed (b). Note the more mature urediniospores (us) are located towards the centre of the uredinium. 7 dpi. Bar = 10 μm.

Fig. 45. Developing urediniospores at different stages of pigmentation. Note the brightly fluorescing empty pedicels (p). 8 dpi. Bar = 20 μm.

Fig. 46. Characteristic high dome shape of a maturing uredinium. Note urediniospores with developing pigmentation and the stoma (arrow) in the transparent cuticle above the uredinium. 10 dpi. Bar = 30 μm.



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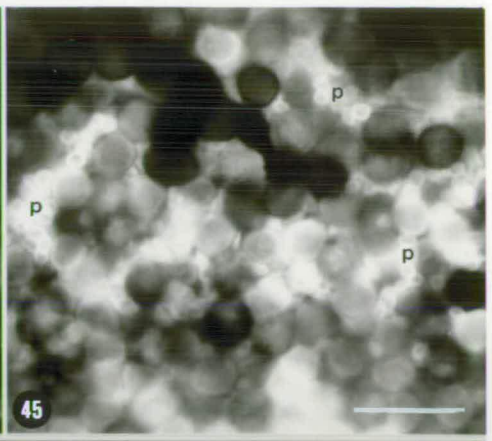

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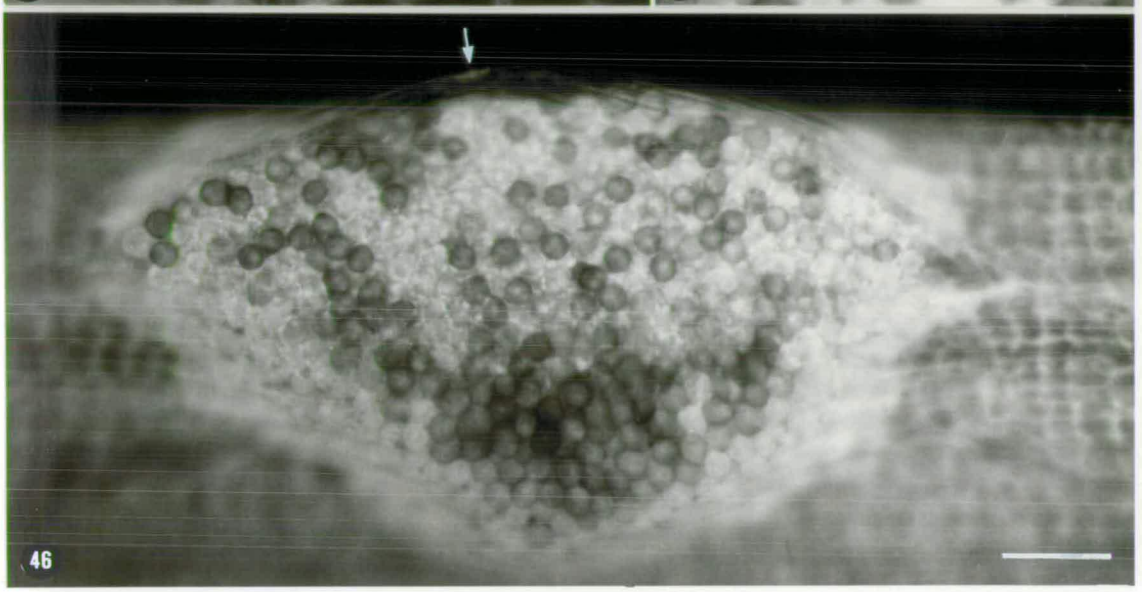

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sub-epidermal tissue subsequently gave rise to urediniospores (Figs. 44, 47, 48). The very young non-pigmented spores at the edge of the uredinium stained intensely with Uvitex (Fig. 44). Longitudinal fractures through uredinia revealed new spores, elongated in shape and without spines, developing from the uredinial bed (Figs. 47, 48). The spores matured at the ends of pedicels which became increasingly club-shaped (Figs. 47, 48). As the spores developed, spines became evident on their surfaces (Figs. 47, 48). Elongation of the pedicels pushed the spores upwards to the top of the uredinium where they became detached when fully turgid (Fig. 48). From above, the pedicels fluoresced intensely amongst the virtually non-fluorescing, mature pigmented spores. In this respect pedicels resembled paraphyses (Fig. 45).

The pressure exerted by the repeated formation of new spores toward the centre probably caused the epidermis to separate from the mesophyll layer to form a high-domed uredinium (Figs. 46, 50, 51). The uredinium split laterally to reveal mature, echinulate spores beneath the site of penetration, where the original infecting spore, germ tube and appressorium were often still in place (Fig. 49). With continued spore formation, the uredinium became more prominent as the opening in the epidermis widened (Figs. 50, 51).

Continued radial growth of the colony gave a dense mycelial network which grew around areas of necrosis from earlier resistance reactions (Figs. 52, 53). Hyphae that entered these areas rapidly stopped growing. Hyphal aggregations appeared within individual colonies and developed into secondary uredinia which frequently merged (Fig. 54) forming a broken ring of uredinia around the primary uredinium.

4.3. Discussion

The primary germ tube of *P. hordei* typically grew across the long axes of barley leaves at right angles to the epidermal cell junctions. This orientated growth pattern has also been shown previously by *P. hordei* on barley leaves (Clifford, 1972; Clifford *et al.*, 1985; Niks, 1981; Read *et al.*, 1992). In addition, *P. hordei* germ tubes will exhibit directional growth towards stomata on a graminaceous non-host (wheat) (Niks, 1981). This suggests that the topography associated with cell junctions provides signals for directional growth of *P. hordei* germ tubes towards stomata. The directional growth pattern of this fungus has been mimicked on artificial microfabricated topographies indicating that it is a contact-mediated

Figs. 47-51. *P. hordei*/barley leaf
Scanning electron micrographs of the reproductive phase

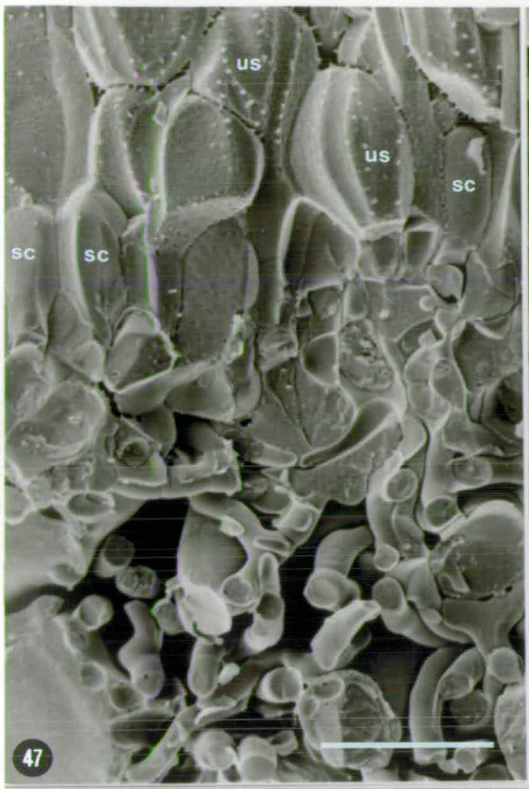
Fig. 47. Fracture through the uredinial bed showing the hyphae which make up the layer of multi-septate fungal tissue. Note the clavate sporogenous cells (sc) and the young echinulate urediniospores (us). 9 dpi. Fully frozen-hydrated. Bar = 20 μm.

Fig. 48. Fracture through another region of the uredinium in Fig. 47 showing elongated pedicels (p) and detached mature ovate urediniospores (us) towards the top of the uredinium. Note the apex of a pedicel (arrow). The urediniospore, which was borne on this pedicel, has been removed during fracturing. 9 dpi. Fully frozen-hydrated. Bar = 20 μm.

Fig. 49. Surface view of the barley leaf showing mature urediniospores emerging through a longitudinal split in the anticlinal walls of the epidermal cells. Note the collapsed urediniospore (us), the germ tube (g) and the appressorium (a) located over the stoma. These infection structures gave rise to the colony from which the underlying uredinium developed. 7 dpi. Partially freeze-dried. Bar = 40 μm.

Fig. 50. Mature urediniospores being released at the surface of the uredinium. Note the absence of paraphyses. 7 dpi. Partially freeze-dried. Bar = 80 μm.

Fig. 51. The epidermis and cuticle peeled back to accommodate the expanding uredinium. Note the smaller urediniospores (arrows) at the edge of the uredinium and the collapsed urediniospores of the original inoculum (asterisks). 7 dpi. Bar = 70 μm.



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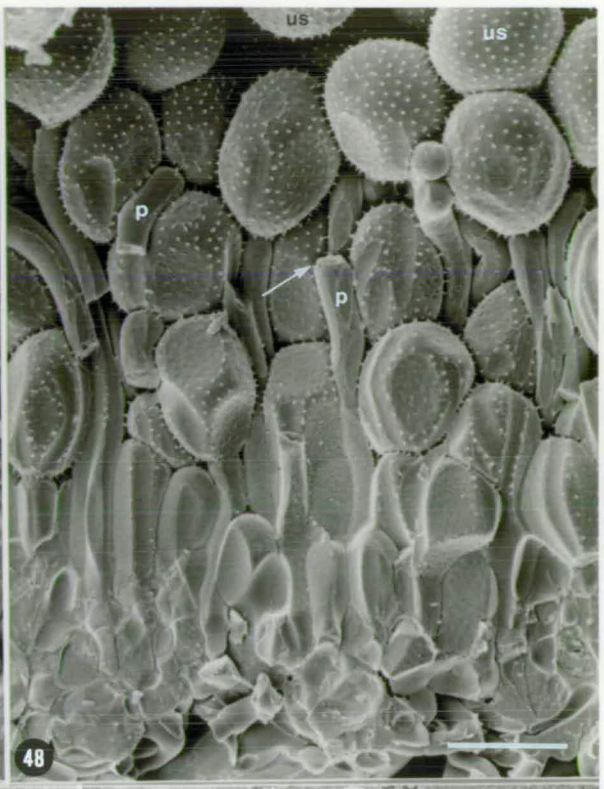

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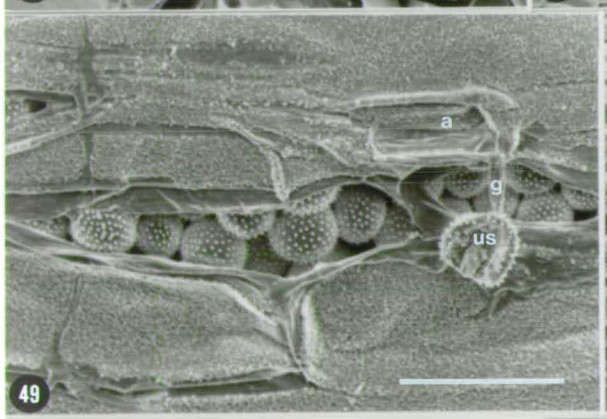

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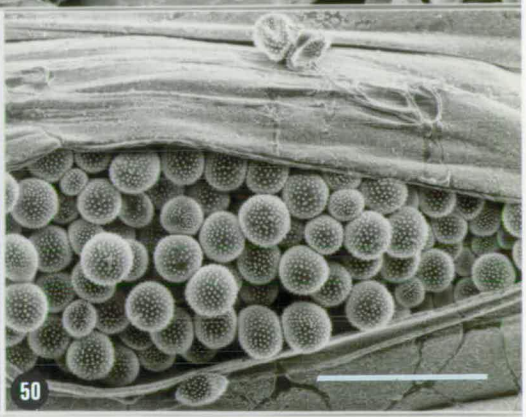

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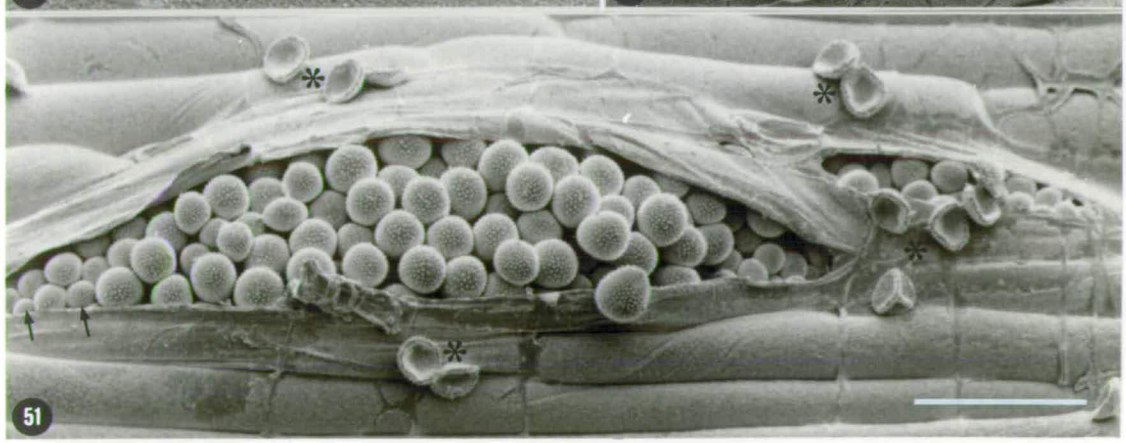

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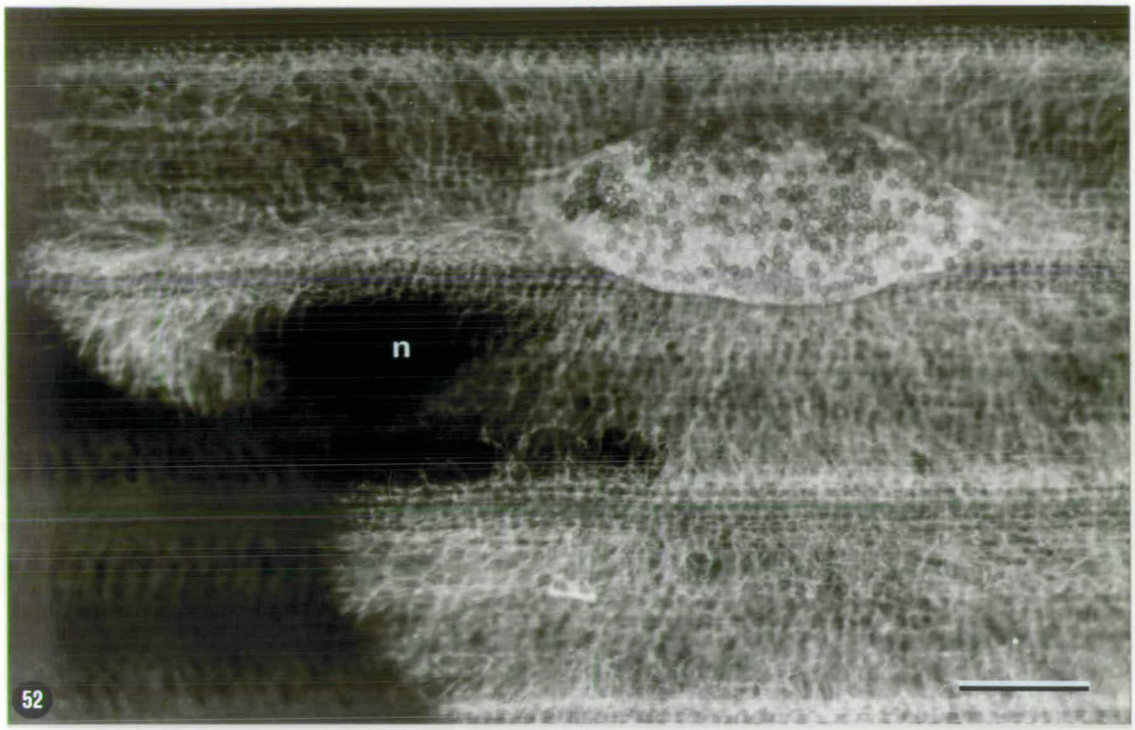
Figs. 52-54. *P. hordei*/barley leaf
Fluorescence micrographs of established infections

Fig. 52. Maturing uredinium at the centre of a colony. Note the cessation of growth in a region of necrosis (n) at an earlier aborted infection site. 7 dpi. Bar = 100 μm.

Fig. 53. Growth around the site of an earlier aborted infection of which the appressorium (a_1) can still be seen. Note the second appressorium (a_2) from which the underlying uredinium ultimately arose. 15 dpi. Bar = 50 μm.

Fig. 54. Low power view of the infected leaf area (asterisk) in Fig. 53 showing secondary uredinial beds (b) and secondary uredinia (u). 15 dpi. Bar = 100 μm.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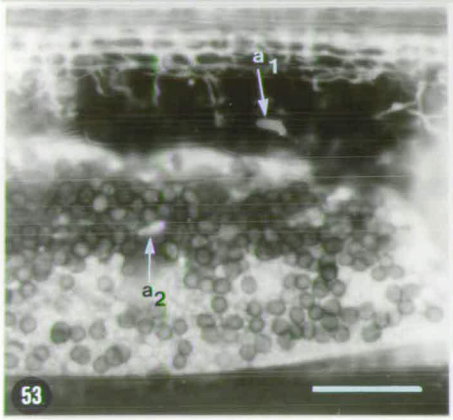
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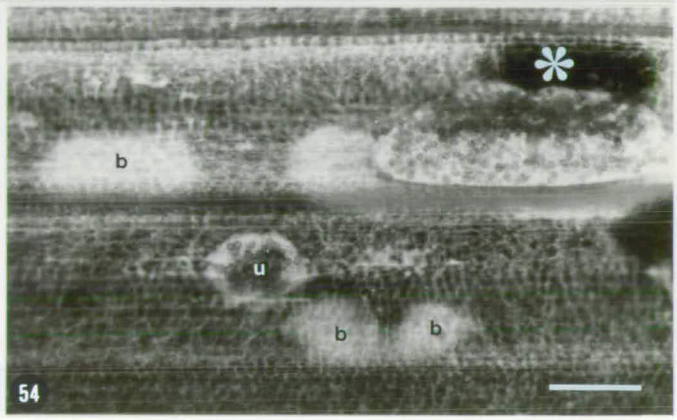
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response (Fig. 64 in chapter 7; Read *et al.*, 1992). Germ tubes of *P. hordei* have also been shown to exhibit a 'nose-down' morphology and to produce an extracellular matrix on their undersides when growing over these artificial surfaces. These observations clearly indicate that the germ tubes are undergoing contact-sensing (Read *et al.*, 1992).

Small lateral side branches usually developed from the primary germ tube over epidermal cell wall junctions. Whether these branches are induced to form, and then guided, as a result of topographical or chemical signals, is not known. Similarly, it is not clear what type of signals are guiding these branches along the junctions. In these potentially "leaky" areas of the leaf surface there may be exudates (Hoch & Staples, 1991), a higher humidity or even free water, and these factors could operate as chemotropic stimuli (Staples *et al.*, 1983). Lateral germ tube branches of *P. hordei* have also been observed to grow along furrows between ridges on microfabricated topographies suggesting a thigmotropic response of a different nature to that of the primary germ tube (Read, N.D. & Kellock, L.J., unpublished results). It should be noted, however, that on leaves, branches sometimes formed over epidermal cells away from cell junctions. On the leaf surface, the short branches are probably exploratory extensions, usually arrested early, but which are sometimes successful in finding a stoma (Fig. 32).

Topographical signals also play a role in appressorium formation by *P. hordei* and this is discussed in detail in chapter 7.

As is typical of morphologically distinct appressoria, the appressorium of *P. hordei* is delimited from the germ tube by a septum. In rust fungi, the septum demarcating the germ tube from the appressorium is thought to have a specific role in the function of the latter infection structure. Unlike the septa found in the intercellular vegetative hyphae, ultrastructural studies have shown that the mature appressorial septum has no central pore (Littlefield & Heath, 1979). This prevents the movement of cytoplasmic material back into the emptied germ tube (Kwon & Hoch, 1991). A non-perforate septum may also help to maintain an increase in turgor during the 'maturation' period of the appressorium (Hoch & Staples, 1991; Terhune *et al.*, 1991) which may have a role in facilitating growth of the penetration peg through the stoma, particularly if the latter is closed.

Infection was often aborted, and this occurred at one of three stages in the susceptible host-pathogen combination analysed here. Abortion occurred during the formation of appressoria, vesicles or haustorial mother cells. Abortion of the latter often resulted in unusually lobed haustorial mother cells (Fig. 37) which has also been previously observed in partially resistant hosts (Niks, 1983*b*). All three

stages of abortion were frequently accompanied by host-cell necrosis, as recognised by bright yellow/brown autofluorescence of the cell wall due to the presence of lignin (Tiburzy & Reisener, 1985). Necrotic areas were inhibitory to later hyphal invasion. Aborted infection in a susceptible barley/*P. hordei* combination has also been observed by Niks (1982) and Parlevliet & Kievit (1986). An explanation for their occurrence may be that host tissue is a genetic mosaic with various levels of resistance distributed at random throughout the leaf (Niks, 1990). In my observations, the fungus was able to compensate to some extent when these reactions occurred: where growth at one end of the vesicle was arrested, a colony would eventually develop from the other end.

The uredinia of *P. hordei* were typically high-domed, a feature which appeared to be due in part to the presence of long pedicels in the uredinium. The pedicels lacking spores resembled paraphyses when viewed from above. Often in the past, old, usually elongated pedicels were described as paraphyses. However, true paraphyses, if they occur in the uredinium, develop before the first urediniospores (Wilson & Henderson, 1966). Of the cereal rusts, only the uredinia of *P. coronata* are thought to contain paraphyses (Harder, 1984).

Where infection sites were at adjacent substomatal cavities, the primary uredinia would sometimes merge but, more typically, would remain discrete in the centre of the densely branched colony. The mycelium of the colony corresponded to an area of chlorosis seen macroscopically around the primary uredinium. In older infections, the depleted uredinia were in the centre of green islands in chlorotic leaf tissue. Green islands in the barley/brown rust system are thought to result from the synthesis and/or retention of chlorophyll in the colony (Farrar & Lewis, 1987; Scholes & Farrar, 1987). The prolonged presence of chlorophyll, within host cells surrounded by the mycelium, probably allows the continued provision of nutrients to the fungus via photosythesis. This may then permit the continued sporulation of secondary uredinia around the mature primary uredinium.

5. QUANTIFICATION OF THE CONTRASTING INFECTION STRATEGIES OF BROWN RUST AND YELLOW RUST OF BARLEY

5.1. Introduction

P. striiformis and *P. hordei* share similar taxonomic characters with respect to urediniospore, teliospore and telium morphologies, and as a result have been grouped together in the "*P. recondita* lineage" (Savile, 1984). Yellow rust contains two subgroups: *P. striiformis* f. sp. *hordei* and *P. striiformis* f. sp. *tritici* which primarily infect barley and wheat, respectively. The *P. recondita* group also contains *P. coronata* (the causal agent of crown rust of oats) and *P. recondita* f. sp. *recondita* and *P. recondita* f. sp. *tritici* which cause the brown rusts (syn. leaf rusts) of rye and wheat, respectively (see Table 2). Although grouped together, *P. striiformis* f. sp. *hordei* and *P. hordei* develop morphologically dissimilar infection structures and exhibit contrasting courses of infection (see chapters 3 and 4).

This chapter provides a quantitative study of infection development by yellow and brown leaf rusts on the same barley cultivar (Golden Promise). Percentage germination, germ tube lengths, number of penetration events, colony size, uredinial number, size of infected area and spore production during infection are analysed because these are common parameters used to measure levels of rust disease (e.g. see: Teng & Close, 1977, 1978; Clifford & Roderick, 1981; Kellock & Lennard, 1982; Falahati-Rastegar *et al.*, 1983; Schlegel & Opel, 1983; Wolffgang & Meister, 1984; Casulli, 1985; McGregor & Manners, 1985; Osman-Ghani & Manners, 1985). The data were analysed using analysis of variance (anova) computed with Genstat. This anova statistics program can analyse up to three treatments (three-way analysis) in an experiment, and gives standard errors of differences of means for calculation of significant differences between means.

5.2. Results

5.2.1. Germination, germ tube lengths and penetration events (Table 3)

48 h after inoculation of leaves, percentage urediniospore germination was 74.5% for barley yellow rust (BYR) and 76.2% for barley brown rust (BBR) which was not significantly different. The percentage of germ tubes which penetrated stomata was 60.1% for BYR (= 44.8 penetration events per cm^2 of segments from the mid

Table 3. Percentage germination, lengths of primary germ tubes (both undifferentiated and differentiated/penetrated) and number of penetration events of *Puccinia hordei* and *P. striiformis* f. sp. *hordei* on barley leaves after 48 h post inoculation.

Pathogen	Percentage germination	Germ tube length(μm)	Penetration events/cm^2
Puccinia striiformis f. sp. *hordei*	74.5	631	44.8
Puccinia hordei	76.2	289	50.5
s.e.d. ±	(df=399)3.89	62.7(df=39)	8.68(df=7)

Table 4. Number of urediniospores per mg of non-hydrated spores, mean weights of individual urediniospores and total urediniospore number per infected leaf for *Puccinia hordei* and *P. striiformis* f. sp. *hordei*.

Pathogen	Spore number per mg non-hydrated spores	Mean weights (ng) of individual non-hydrated spores	Total spore number per infected leaf
Puccinia hordei	86,200	12.161	699,341
Puccinia striiformis f. sp. *hordei*	91,733	11.316	1,046,857
s.e.d. ± (df=38)	5,069	0.612	-

region of the leaf) and 66.3% for BBR (= 50.5 penetration events per cm^2 of segments from the mid region of the leaf). Again there was no significant difference between the two pathogens. In contrast, the lengths of the primary germ tubes were significantly different ($p \leq 0.001$) between the two species, those of BYR having a mean length which was over twice as long (631 μm) as that of BBR (289 μm).

5.2.2. Colony size

Changes in size of discrete colonies for each of the pathogens are shown in Fig. 55. Generally, the lengths of colonies were greater than their widths and colony length was greatest down the length of the infected leaf. Colony lengths increased faster with age than widths: this was significant (*t*-test) at 1 dpi and thereafter for BBR ($p \leq 0.001$) and at 6 dpi and thereafter for BYR ($p \leq 0.05$). Increase in width was inhibited in both species by the presence of leaf veins (Figs. 17, 42). Colonies of BBR grew beyond the substomatal cavity 2-3 dpi. In contrast, those of BYR did not invade host tissue surrounding the substomatal cavity until 4-5 dpi. The lengths and widths of colonies of BBR increased in an approximately linear fashion until 8 dpi. The mean colony extension rates (i.e. along colony radii) during this period were approximately 50 μm/day across the leaf and 87 μm/day along the length of the leaf. After 8 dpi neighbouring colonies frequently merged preventing further quantification. In contrast, colony lengths and widths of BYR increased more-or-less linearly for the first 7 dpi and then increased at an approximately exponential rate. The mean colony extension rates during the first 7 dpi were approximately 15 μm/day across the leaf and 19 μm/day along the length of the leaf. Thereafter, the colonies showed a dramatic increase in growth rate up to 216 μm/day across the leaf and 336 μm/day along the length of the leaf. Fusion of colonies prevented further measurements of BYR after 9 dpi.

5.2.3. Uredinial numbers

Uredinia of both species were formed primarily on the adaxial leaf surface. Overall, BYR formed considerably more uredinia per seedling leaf (approximately 856) than did BBR (approximately 171) and produced them over a longer period (over 22 days for BYR as opposed to 16 days for BBR) (Fig. 56*a*). The uredinia of BBR began to sporulate earlier between 6 and 8 dpi as compared with 11 dpi for BYR. The number of sporulating BBR uredinia increased significantly ($p \leq 0.01$) < 8-10 dpi but thereafter remained relatively constant until 18-20 dpi after which they started to significantly ($p \leq 0.05$) decline. After 14 dpi a few new uredinia

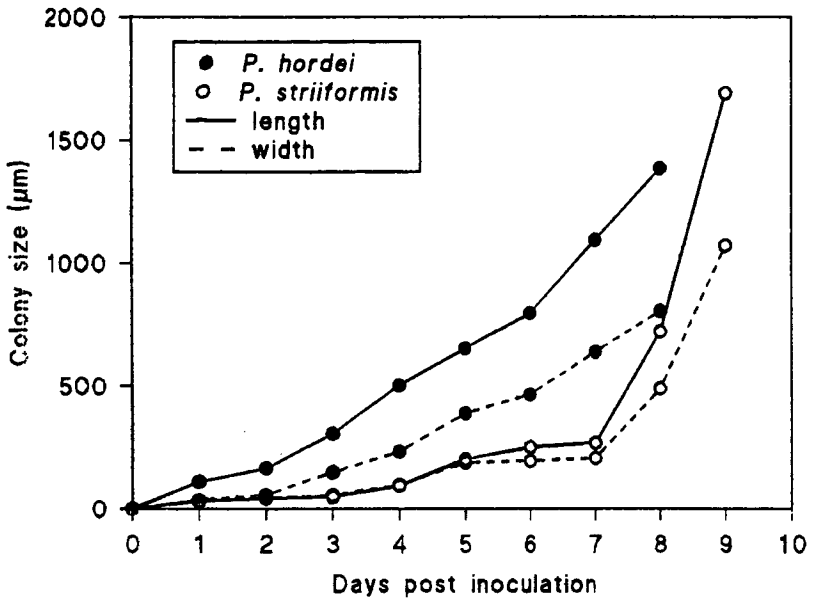


Fig. 55. Growth of discrete colonies of brown rust and yellow rust within barley leaves.

P. hordei: width, s.e.d. = 31.2 (df=133); length, s.e.d. = 46.6 (df=133).

P. striiformis f. sp. *hordei*: width, s.e.d. = 62.6 (df=152); length, s.e.d. = 99.3 (df=152).

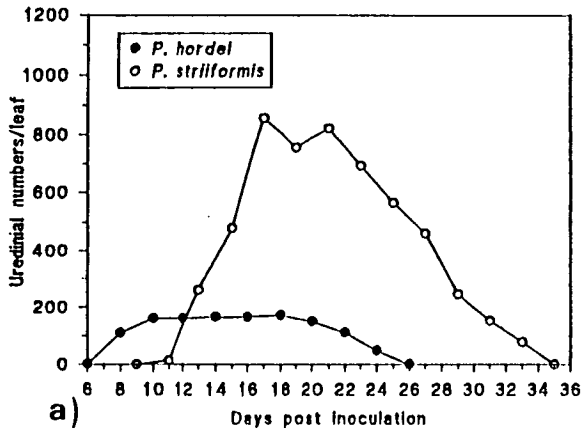
Fig. 56. Sporulation of brown rust and yellow rust on barley seedling leaves.

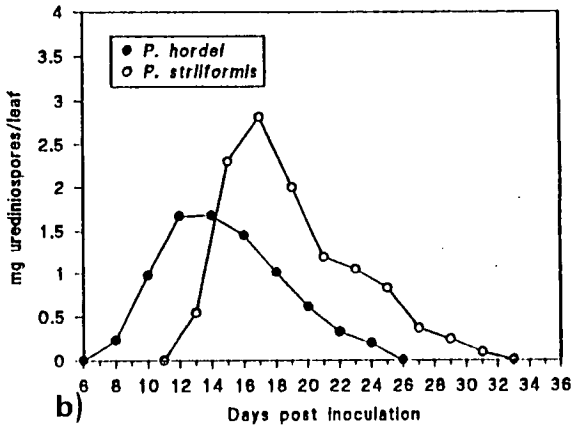
Fig. 56*(a)*. Numbers of sporulating uredinia per leaf
BBR: s.e.d.= 14.3 (df=24); BYR: s.e.d.= 55.8 (df=33).

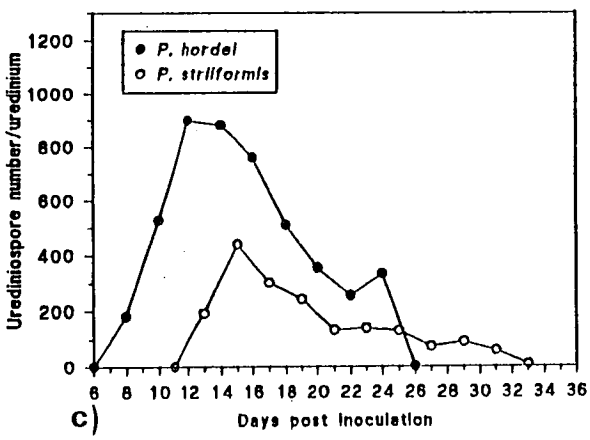
Fig. 56*(b)*. Urediniospore production per leaf
BBR: s.e.d.= 0.11 (df=146); BYR: s.e.d.= 0.12 (df=204).

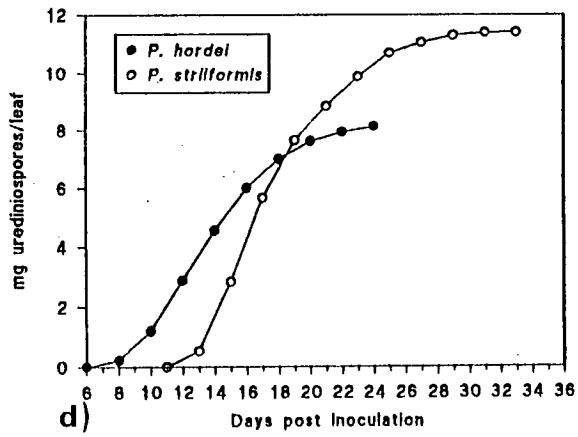
Fig. 56(*c*). Urediniospore numbers per uredinium

Fig. 56(*d*). Cumulative urediniospore weight per leaf


P. hordei
P. striiformis
Uredinial numbers/leaf
0
200
400
600
800
1000
1200
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Days post inoculation
a)


P. hordei
P. striiformis
mg urediniospores/leaf
0
0.5
1
1.5
2
2.5
3
3.5
4
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Days post inoculation
b)


P. hordei
P. striiformis
Urediniospore number/uredinium
0
200
400
600
800
1000
1200
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Days post inoculation
c)


P. hordei
P. striiformis
mg urediniospores/leaf
0
2
4
6
8
10
12
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Days post inoculation
d)

had appeared in leaf tissue at the base of the leaf. In comparison, the number of active BYR uredinia increased dramatically (significant at $p \leq 0.01$ to $p \leq 0.001$ levels) over the first 6 days of formation (11-17 dpi, Fig. 56*a*) as colonies rapidly spread into uninfected leaf tissue (Fig. 59 in chapter 6), particularly towards the base of the leaf after 14 dpi. After 21 dpi, the number of sporulating BYR uredinia began to significantly decline ($p \leq 0.05$) as the tip of the leaf started to senesce with a consequent loss in uredinial viability. Nevertheless, even at this stage, uredinia of BYR became active in new leaf tissue which had appeared by 4 dpi at the base of the leaf (see section 5.2.5).

5.2.4. Spore production

The temporal trend of spore production by the two pathogens approximately mimicked uredinial number except that the decline in spore production occurred 4 days earlier than the reduction in uredinial number (cf. Figs. 56*a* and 56*b*). Overall, BYR produced more spores per leaf (11.412 mg after 33 days) than did BBR (8.113 mg after 24 days) although cumulative spore production by BYR < 18 dpi lagged behind that of BBR (Fig. 56*d*). From visual assessments, the only spores produced by BBR after 22 dpi were from secondary uredinia formed around the exhausted primary uredinia. The significant decline ($P \leq 0.001$) in BYR spore production from 17 to 21 dpi (Fig. 56*b*) reflected the declining activity of uredinia at the senescing leaf tip. However, from 21 to 25 dpi, spores were released from new uredinia in host tissue towards the base of the leaf into which the pathogen had subsequently spread.

The number of spores per mg of non-hydrated spores was calculated (see section 2.3.2.3) and found to be 86,200 for BBR and 91,733 for BYR, which were not significantly different (Table 4). From these data for each species, the mean weights of individual spores were calculated by Genstat as 12.161 ng for BBR and 11.316 ng for BYR which were also not significantly different (Table 4). The total number of spores produced from an infected average seedling leaf (see section 5.2.5) was also determined (699,341 for BBR and 1,046,857 for BYR) (Table 4). Using these calculated values of the numbers of spores per mg, and the data from Figs. 56*a* and 56*b*, it was possible to calculate the numbers of spores produced per uredinium with time (Fig. 56*c*). This showed that the individual uredinia of BBR were more productive than those of BYR, and that the uredinia of both rusts were most active in the earliest stages of sporulation (< 12 dpi for BBR and < 15 dpi for BYR). The rise in the numbers of spores produced per uredinium by BBR between 22 and 24 dpi, probably resulted from the freshly sporulating uredinia

seen in the younger leaf tissue which became exposed towards the base of the leaf between inoculation and 4 dpi (see section 5.2.5). These uredinia probably arose from infections by local urediniospores from the initial uredinia.

5.2.5. Length of leaf infected

All the first true leaves exhibited an erect growth habit and the mean (df=10) lengths of the leaves at the time of inoculation with BYR and BBR in the settling tower were 148.2 mm (s.e. 3.7 mm) and 154.8 mm (s.e. 4.94 mm) respectively (Table 5). These leaf sizes were not significantly different from each other. At this growth stage, the ligules had differentiated at the base of the leaves. After inoculation, elongation of the leaf sheath continued and the ligule, and some previously unexposed leaf tissue (approximately 4 mm long), appeared at the base by 4 dpi (Table 5).

The maximum relative length of leaf that became infected was slightly less for BBR than for BYR (10 mm remained uninfected by BBR as compared with only 4 mm for BYR) and this was attained much earlier by BBR (10 dpi) than for BYR (15 dpi) (Table 5). Both rusts maintained the maximum sporulating length of leaf for 10 days. Apart from the delay by BYR in the appearance of sporulating uredinia (Fig. 56*a, b*), the subsequent length of time that the leaves supported sporulation was the main distinguishing feature between these two rusts (4 days for BBR and 8 days for BYR). This was due to the delayed senescence in leaves at the lower temperature optima for BYR infection. At the end of BBR sporulation (between 24-26 dpi, Fig. 56*b*) only 30 mm green leaf remained. Before the end of BYR sporulation, at 24 dpi 63.4 mm of green leaf remained and this was reduced to 26.5 mm at 33 dpi when sporulation finished (Fig. 56*b*). This longer period of green leaf retention in BYR infections suited the semi-systemic growth habit of BYR which was able to continue extending towards the leaf base as senescence progressed from the tip.

5.2.6. Urediniospore morphology and dimensions

Urediniospores released from uredinia of both species were naturally dehydrated and collapsed (Fig. 58). After hydration, by placing them in distilled water they immediately swelled up and acquired a roughly ovoid morphology in which their lengths were greater than their widths (Fig. 57). The hydrated spores of brown rust were slightly larger than those of yellow rust, with small, but statistically significant differences in both length ($p \leq 0.01$) and width ($p \leq 0.05$) (Table 6).

Table 5. Temporal changes in length of green leaf and length of leaf exhibiting sporulation.

	Mean leaf length when inoculated (mm)	max. length of green leaf (mm) and dpi when this was reached (in brackets)	max. length (mm) of leaf exhibiting sporulation and dpi when this was reached (in brackets)	Length (mm) of green leaf remaining at end of sporulation and dpi when this was reached (in brackets)
BBR	154.8	158.9 (4)	149.4 (10)	30.0 (24)
BYR	148.2	152.0 (4)	147.9 (15)	26.5 (33)

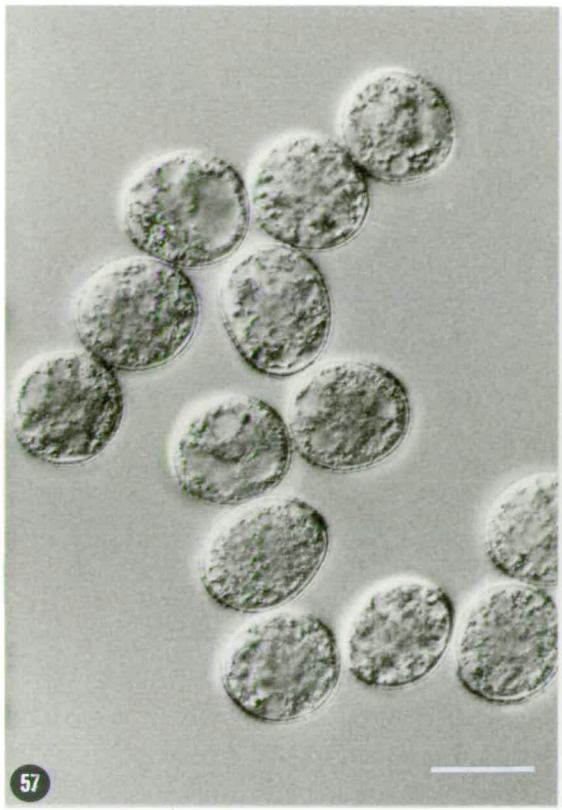
Figs. 57, 58. Differential interference light micrographs of urediniospores of *P. striiformis* f. sp. *hordei*.

Fig. 57. Hydrated urediniospores in distilled water
Bar = 20 μm.

Fig. 58. Non-hydrated urediniospores in cottonseed oil
Bar = 20 μm.

Table 6. Sizes of hydrated urediniospores of *Puccinia hordei* and *P. striiformis* f. sp. *hordei*.

Pathogen	length(μm)	width(μm)
Puccinia hordei	26.83	21.663
Puccinia striiformis f. sp. *hordei*	25.86	21.244
s.e.d. ± (df=198)	0.3	0.171



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58

5.3. Discussion

Quantitative assessments of developmental stages in the infection strategy of each rust revealed many differences, and these are dealt with in detail below.

5.3.1. Percentage germination, germ tube lengths and penetration

The percentage germination of BYR and BBR on the leaf surface was relatively high (approximately 75% in both cases). On susceptible seedling leaves, at 4-5°C, the percentage germination of urediniospores of wheat yellow rust (WYR) has been recorded as varying from 3-98% by different authors (Sharp, 1965; Stubbs & Plotnikova, 1972; Russell, 1976; Cartwright & Russell, 1981; Helfer, 1986; Rubiales & Niks, 1992). Published figures for BYR are not available. BBR, on the other hand, has been shown to exhibit 64-90% germination on seedling leaves at temperatures between 18 and 20°C (Simkin & Wheeler, 1974; Falahati-Rastegar *et al.*, 1983; Helfer, 1986; Rubiales & Niks, 1992). It should be noted, however, that most germination studies on urediniospores have been conducted on agar (e.g. for *P. striiformis* see: Manners, 1950; Tollenaar & Houston, 1966; Maddison & Manners, 1972; Gopalan & Manners, 1984; Opel *et al.*, 1986*a*; for BBR see: Helfer, 1986) and the percentage germination on this medium is usually greater than that given above e.g. 90-100% for WYR (Gopalan & Manners, 1984). On the leaf surface many factors can influence the percentage germination such as the cultivar used, leaf age, leaf position on the plant (Russell, 1976), and possibly phylloplane microflora (Lucas & Knights, 1987).

Although the primary germ tubes of *P. hordei* were, on average, half the length of those of yellow rust, the lateral germ tube branches and other branches would add to their overall length. Longer than average primary germ tubes of *P. hordei* have been correlated with a reduction in the number and size of colonies (Niks, 1990). Comparisons of spore production between the two pathogens showed that the relatively long lengths of BYR germ tubes was not detrimental in this respect. The shorter primary germ tubes of BBR before penetration into the leaf could imply that BBR germ tubes were more successful at recognising stomata than BYR germ tubes. Both the germ tubes of BBR and BYR exhibited directional growth on the leaf surface by growing across the leaf axis (chapters 3 and 4) thereby increasing the probability of encountering stomata on these graminaceous leaves (Read *et al.*, 1992).

The large difference in primary germ tube lengths of the two rusts was not reflected in the percentage penetration of each rust (i.e. 66% for BBR as opposed

to 60% for BYR which were not significantly different). In WYR, percentage penetration on the adaxial surface of susceptible seedling leaves has been reported as varying from 4-28% (Stubbs & Plotnikova, 1972; Russell, 1977; Rubiales & Niks, 1992). Penetration rates of 41% (Rubiales & Niks, 1992) and 92-99% (Falahati-Rastegar *et al.*, 1983) have been recorded for BBR on seedling leaves. The variation in recorded germination and penetration rates emphasises the difficulty in comparing different isolate/cultivar combinations.

5.3.2. Colony size

The time from penetration to when individual colonies merge within the leaf can reflect the severity of the infection. This occurred in BBR and BYR after 8 dpi and 9 dpi, respectively. However, in heavy and light infections, colonies of BBR have been known to merge at 4 dpi and 10 dpi, respectively (Falahati-Rastegar *et al.*, 1983). Thus it would appear that the BBR infections reported here were moderate to light, although comparisons with other cultivar/isolate combinations are difficult to make. In a slow rusting cultivar, Scholes & Farrar (1987) divided the increase in colony area of *P. hordei* into three phases: an initial lag phase 0-3 dpi; rapid linear growth 4-8 dpi; and cessation of growth 9-12 dpi. The approximate rates of hyphal extension during these phases were 54, 100 and 12 μm per day respectively. These growth rates are consistently slower than those recorded here (221 μm per day colony length between 4-8 dpi) for BBR on a more susceptible cultivar.

The colonies of BYR showed a dramatic increase in growth rate after 7 dpi from 59 (4-7 dpi) to 711 μm (7-9 dpi) per day. Measurements of the colonisation rate of yellow rust within wheat leaves would suggest that this growth rate can probably increase further: a rate of about 5 mm per day in sporulating mycelium has been recorded in a susceptible wheat cultivar at 15°C (McGregor & Manners, 1985).

5.3.3. Uredinial number, spore production and length of leaf infected

The erect growth habit of cereal leaves is known to affect spore deposition (Russell, 1975) and thus the regions on the leaf where infection subsequently occurs. In this thesis, the inoculated leaves were all of the same age and growth habit and thus the same leaf areas were exposed to the inoculum in each case.

The latent period (i.e. the time from inoculation to the eruption of sporulating uredinia), was shorter for BBR than BYR. However, unlike BYR, uredinial numbers did not increase dramatically thereafter. Casulli

(1985) found that the latent period of BBR was significantly different in different regions of the leaf: the tips of leaves had a significantly shorter latent period than the mid or basal regions. Furthermore, the tip of the leaf produced spores for a short time only (3-6 days) because this region of the leaf dried early. Early senescence of the leaf tip was also seen here in BBR and BYR infected leaves. The cereal leaf tip is physiologically more mature and is the first part of the leaf to senesce when the leaf dies (Langer, 1972). In leaves infected with BBR, this declining viability of the leaf from tip to base was directly reflected in the reduction of the length of leaf infected. In contrast to BBR, the BYR mycelium continued to extend into basal leaf tissue after the first appearance of pustules, thus maintaining a maximum length of infected leaf longer than for BBR. BBR also produced uredinia in this part of the leaf but only from re-infection, and by this time much of the distal end of the leaf had senesced. A sudden reduction in the leaf length containing actively sporulating uredinia then occurred because of leaf senescence. However, the less advanced senescence of the leaves in the lower optimum temperature for BYR infection, meant that continued uredinial activity towards the base of the leaf maintained spore production for longer than for BBR.

The measurement of spore production proved to be a more reliable indicator of the relative success of each rust pathogen than measurements of the numbers of sporulating uredinia alone. This was because the secondary uredinia of both rusts frequently merged making assessments of cumulative numbers difficult.

Peak spore production by both rusts was reached relatively early in the infectious period (i.e. after 12 dpi for BBR and 17 dpi for BYR). A similar trend in maximum spore production was also noted for BBR by Teng & Close (1978) and for WYR by McGregor & Manners (1985). This also occurs in *P. recondita f. sp. tritici* and *Uromyces phaseoli* (Mehta & Zadoks, 1970; Yarwood, 1961) and in *P. graminis* and *P. coronata* (Kochman & Brown, 1975). The interruption in the rapid rate of decline in sporulation of BYR is similar to the situation in WYR grown at 10°C (McGregor & Manners, 1985), and suggests a lag phase before uredinia are formed at the base of the leaf. This may be related to the rate of colony extension within the leaf which is very temperature dependent. It has been shown, for example, that the spread of WYR colonies within the leaf is increased to a much greater degree by increasing temperature than is the frequency of WYR uredinia formed per unit area (McGregor & Manners, 1985). The greater number of spores produced here by BYR than BBR was a direct consequence of the semi-systemic type of invasion into leaf tissue, which given the respective optimum conditions selected, resulted in an extended period of sporulation.

The number of spores produced per uredinium showed that the uredinia of BBR were more productive and this would compensate to some extent for the fewer numbers of uredinia produced by this rust. The maximum number of spores produced per BBR uredinium in this study was generally higher than those recorded by Teng & Close (1978) on seedling leaves in a susceptible race\cultivar combination. There were more spores per uredinium per day by BYR in this study than from WYR on a susceptible cultivar (McGregor & Manners, 1985). Comparison of their results with the results reported in this thesis are again difficult to make because of differences in the combination of host cultivar and isolate. However, seedling leaves of Golden Promise have been shown to produce a greater total number of BBR spores than recorded here, but a different isolate was used for inoculation (Casulli, 1985).

5.3.4. Urediniospore morphology and dimensions

Urediniospores released from uredinia naturally dehydrate and collapse (Beckett *et al.*, 1984; Read, 1991). It was in this state that the spores of BYR and BBR were maintained and weighed. Upon hydration, such as would occur in conditions suitable for germination, the urediniospores swelled immediately, increasing their volume substantially. Considerable differences between the dimensions of dried (i.e. non-hydrated) and 'wet' (i.e. hydrated) urediniospores of rusts have been previously reported (Strobel, 1965; Knights & Lucas, 1980; Beckett *et al.*, 1984).

The size of the urediniospores of *P. striiformis* and *P. hordei* has been reported to fall within a similar range (Wilson & Henderson, 1966, Savile, 1984). However, in one of these studies (Savile, 1984), specimens were mounted in lactophenol and this may not have permitted full spore expansion on hydration, as would occur in pure water. In this thesis, the urediniospores of the BBR isolate were found to be slightly larger and heavier than those of BYR in the dried state. Consequently, there were more spores per mg of BYR, although the difference was not significant. Because equal inoculum weights were used to infect plants with each rust, BBR inocula contained slightly fewer urediniospores than did BYR. In this study, 86,200 BBR spores per mg were recorded; Teng & Close (1978) reported a figure of 57,470 per mg whereas values of 450,000 and 620,000 spores per mg have been recorded for *P. graminis* f.sp. *tritici* (Rowell, 1984) and 93,900 spores per mg for *Lycopodium* (Gregory, 1951; Sreeramula & Ramalingham, 1961). The differences possibly reflect different states of spore hydration.

5.3.5. Efficiency of the infection strategy of each pathogen

The weight of spores produced has proved to be a very sensitive measure of the variability of infection levels in yellow rust (Johnson & Bowyer, 1974), revealing differences between treatments not shown by measurements of colony size alone (Kellock & Lennard, 1981). In addition, measurements of the maximum size of an infected area supporting active pustules, together with records of total spore production from the same area, can be used to give an estimate of the relative 'efficiency' of the infection strategy of a pathogen. BYR produced **0.0772 mg spores/mm** infected leaf length and BBR yielded **0.0543 mg spores/mm** infected leaf length. Thus, BYR appears to possess the more efficient infection strategy; within one generation, semi-systemic spread through the leaf is more productive than localised spread under the optimum conditions selected. The relative success of each pathogen over a growing season would therefore depend a great deal upon the time from deposition of urediniospores to the first eruption of uredinia that could initiate further cycles of infection and sporulation. This of course is governed by many other factors such as temperature, relative humidity and availability of suitable leaf tissue.

6. A SUMMARY OF THE COMPARISONS OF BARLEY YELLOW RUST AND BARLEY BROWN RUST

6.1. Introduction

A comparative analysis of the different developmental and infection strategies of the two barley rusts has been made using data from chapters 3-5.

6.2. Course of infection

In order to interpret the comparisons of the infection strategies of the two rusts, the course of infection for barley yellow rust (BYR) and barley brown rust (BBR) is shown diagrammatically in Figs. 59 and 60 and brief descriptions are given below.

6.2.1. *P. striiformis* f. sp. *hordei*

Penetration occurs through stomata without the formation of an appressorium. A vesicle and primary infection hyphae develop within the substomatal cavity (Fig. 59*a*). Primary haustoria form in mesophyll cells from terminal haustorial mother cells. Between 2 and 4 dpi, vesicles and primary infection hyphae behind haustorial mother cells become swollen (Fig. 59*b*). From 4 to 9 dpi, the colony expands intercellularly via large aseptate invasive hyphae. Uredinial bed hyphae appear between 9 and 10 dpi and indicate future sites of primary uredinia. This is immediately followed by a "wave" of septation which moves back along the invasive hyphae from the uredinia to the site of penetration and then outwards to the colony periphery (Figs. 59*b*,*c*). By 10-11 dpi, developing urediniospores are evident in the primary uredinia and some start to be released. After 12 dpi, all primary uredinia are actively sporulating whilst secondary uredinia start to develop from numerous spiky hyphae which form at intervals along the, by now, septate runner hyphae (Fig. 59*c*). The runner hyphae extend along the length of the leaf growing into new tissue, particularly towards the proximal end of the leaf. Mature secondary uredinia frequently merge from 16 dpi onwards (Fig. 59*d*) to give long stripes of sporulation. As the tip and middle of the leaf senesce, uredinia are produced in new tissue towards the leaf base during the later stages of infection spread. Sporulation continues until about 32 dpi.

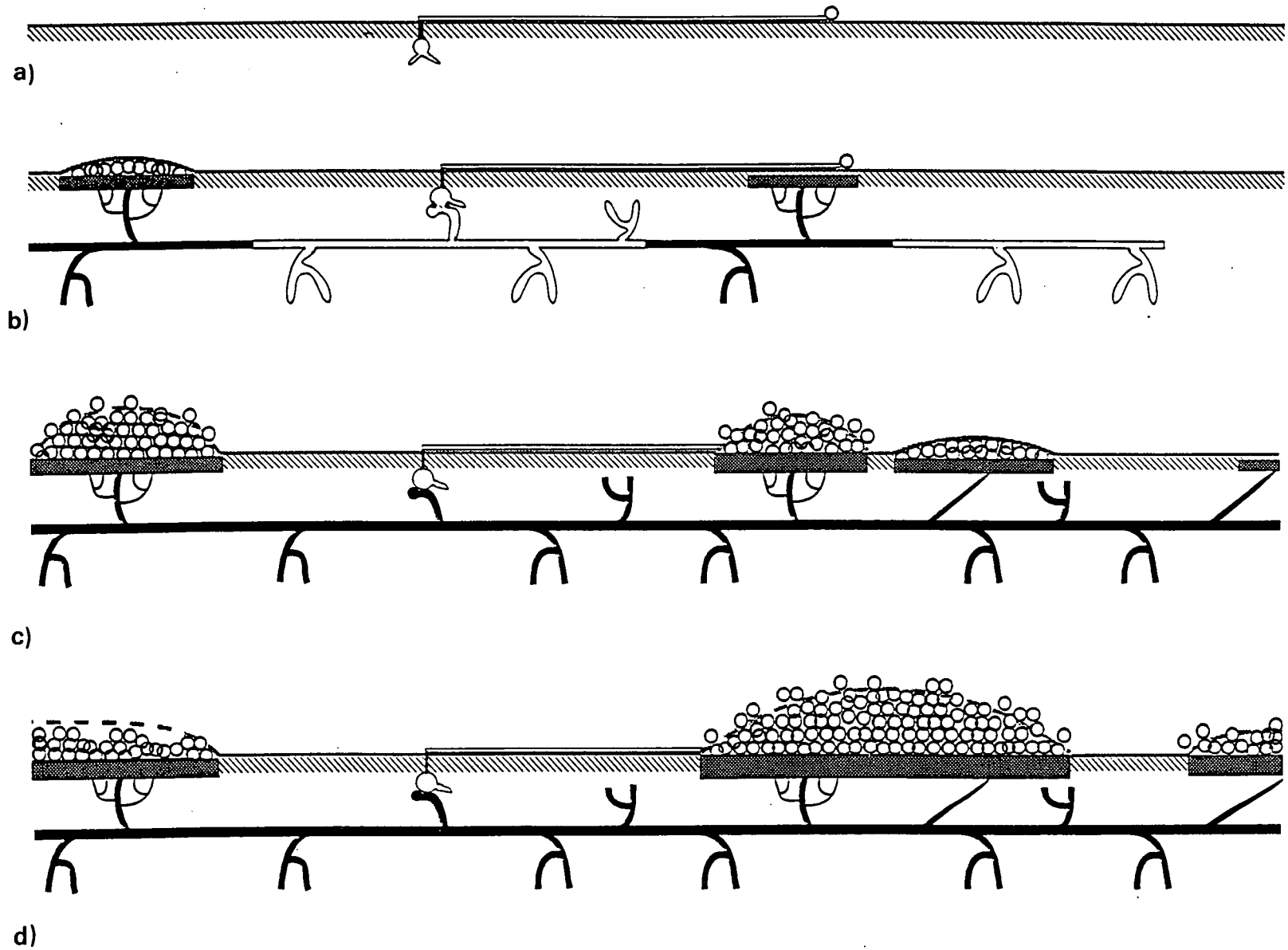
Fig. 59. Diagrammatic representation of the course of infection of yellow rust of barley. (not to scale)
(Key: open fill figures = aseptate; solid fill figures = septate)

Fig. 59(*a*). < 4 dpi. Urediniospore and germ tube with penetration through a stoma occurring without appressorium formation. Development of an aseptate vesicle and two aseptate primary infection hyphae (open figures) within the substomatal cavity.

Fig. 59(*b*) 4-10 dpi. The swollen vesicle and primary infection hypha from which large, branching aseptate runner hypha (open figure) have developed. Thin branches from the runner hypha form the primary uredinia some distance from the penetration site. Septation (solid fill) develops back from areas of reproduction towards the site of penetration and then outwards to the colony periphery.

Fig. 59(*c*) 10-12 dpi. Primary uredinia start to sporulate whilst secondary uredinia are initiated from 'spiky' hyphae on the septate, runner hyphae.

Fig. 59(*d*). 12-16 dpi. Uredinia merge at intervals along the long axis of the leaf to give the stripes of sporulating uredinia characteristic of yellow rust.


a)
b)
c)
d)

6.2.2. *P. hordei*

Penetration occurs through a stoma after the formation of a morphologically distinct appressorium. A vesicle, orientated parallel to the long axis of the leaf, develops in the substomatal cavity within the first 24 h along with typically bipolar infection hyphae (Fig. 60*a*), terminal haustorial mother cells and accompanying haustoria. During 2-3 dpi, branching septate hyphae spread out at either end of the vesicle giving the young colony a 'bow tie' appearance (Fig. 60*b*). Between 4 and 5 dpi, the hyphae under the site of penetration form a subepidermal multi-septate layer of coherent fungal tissue which gives rise to the primary uredinial bed (Fig. 60*c*). By 6 dpi, young urediniospores are apparent in the primary uredinium and the spores are released when the uredinium matures at around 7-8 dpi (Fig. 60*d*). The colony continues to expand radially, and from 10 dpi secondary uredinia develop from hyphal aggregations in the expanding colony and frequently merge to form a broken concentric ring of uredinia around the primary uredinium (Fig. 60*e*). Sporulation continues until about 25 dpi.

6.3. Contrasting features

Thirteen contrasting features of the two rusts are summarised in Table 7. Each feature is discussed in the following sections.

6.3.1. Urediniospores

Barley yellow rust urediniospore walls fluoresced brightly when stained with Uvitex whereas mature BBR urediniospores did not. It was also noted that, as the spores of BBR matured in the uredinium, they lost their affinity for the fluorescent dye (chapter 4). This differential staining of urediniospores of the two rusts was also observed by Opel & Lausch (1987) and may be explained by the presence or absence of pigment in the urediniospore wall: the pigment in yellow rust urediniospores is in the cytoplasm (Allen, 1928; Savile, 1984) whilst that of BBR is present in the wall (Savile, 1984). Pigment incorporated into the spore wall probably inhibits the fluorescent brightener from binding to ß-glucans, and/or chitin. Pigment in urediniospores may be important in determining their ability to survive exposure to sunlight because a loss of cytoplasmic or wall pigment is associated with a loss of viability (Maddison & Manners, 1972; Teng & Close, 1981). Furthermore, yellow rust urediniospores are more sensitive to sunlight and lose colour more rapidly than *P. recondita* or *P. graminis* urediniospores which have pigmented walls (Maddison & Manners, 1972). A feature often used to

Fig. 60. Diagrammatic representation of the course of infection of brown rust of barley. (not to scale)

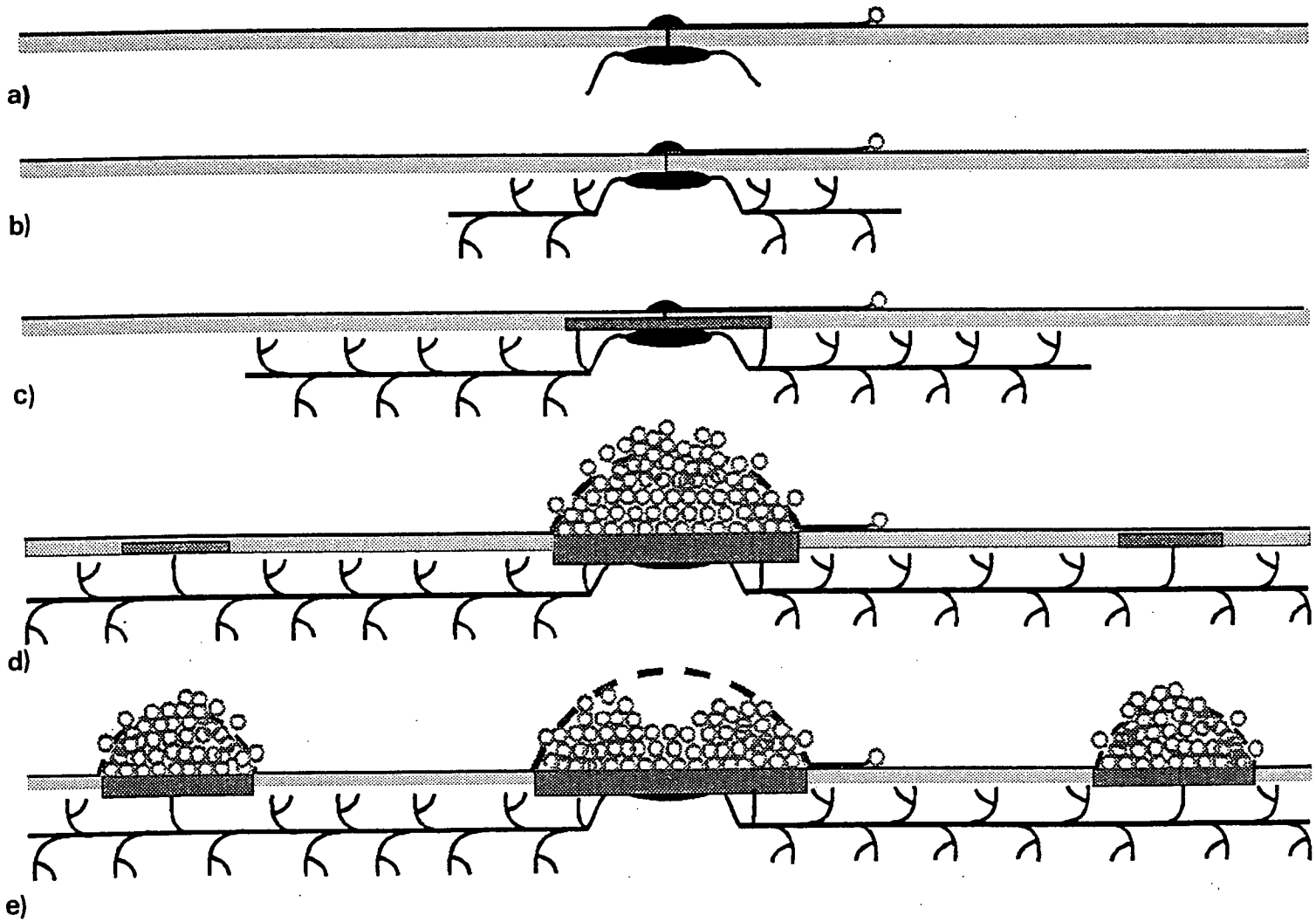
Fig. 60(*a*). 0-24 hpi. Urediniospore, germ tube and appressorium formation. A septate vesicle with bipolar infection hyphae develop parallel to the long axis of the leaf.

Fig. 60(*b*). 2-3 dpi. Branching septate hyphae spread out at either end of the vesicle.

Fig. 60(*c*). 4-5 dpi. Development of the primary uredinial bed from the subepidermal multi-septate layer of coherent fungal tissue under the site of penetration.

Fig. 60(*d*). 7-10 dpi. Sporulating primary uredinium and the development of the secondary uredinial beds in the surrounding mycelium.

Fig. 60(*e*). 12-25 dpi. Secondary uredinia start to sporulate while others continue to develop within the mycelium of the colony. Note that the secondary uredinia often merge to form broken concentric rings of uredinia around the site of penetration.


a)
b)
c)
d)
e)

	BROWN RUST	YELLOW RUST
1.	Pigmented urediniospore wall[2]	Hyaline urediniospore wall[1]
2.	Primary germ tube short[3] with numerous lateral branches	Primary germ tube long[3] and typically without lateral branches
3.	Appressoria formed[2]	Appressoria absent[1]
4.	Elongated, septate vesicle	Globose, aseptate vesicle
5.	Medium-sized[2], septate invasive hyphae	Large[1], aseptate invasive hyphae
6.	No delay in colony spread after penetration[2,3]	Delay in colony spread after penetration
7.	Dense mycelial growth in a radial colony[2,3]	Diffuse mycelial growth in an irregular expansive colony[1,3]
8.	No obvious specialisation of hyphae for invasive growth and uredinial bed formation[2,3]	Specialised hyphae for invasive growth and uredinial bed formation[1]
9.	Primary uredinium under penetration site[2,3]	Primary uredinium some distance from penetration site[1,3]
10.	Long pedicels in uredinium[2]	Very short pedicels in uredinium[1]
11.	Secondary uredinia produced around primary uredinium[2,3]	Secondary uredinia produced along leaf length[1,3]
12.	Relatively short period of spore production[3]	Prolonged period of spore production[3]
13.	Wide optimum temperature range for growth and sporulation[4]	Lower optimum temperature range for growth and sporulation[5]

distinguish the urediniospores of different species of rusts from each other is germ pore number. However, both BBR and BYR urediniospores possess 8-10 germ pores (Wilson & Henderson, 1966).

6.3.2. Germ tube length and lateral branch formation

The germ tubes of both rusts exhibited directional growth across the long axis of the leaf but germ tubes of BYR were over twice as long as the primary germ tubes of BBR. However, BYR germ tubes did not form lateral germ tube branches or show any of the branching typical of BBR germ tubes. In addition, unlike the germ tubes of BBR, the BYR germ tubes often grew over stomata before finally penetrating. However, this did not result in a significantly reduced penetration rate.

6.3.3. Appressorium formation

BBR, in accord with the majority of rust fungi (Allen *et al.*, 1991*a*), always forms a morphologically distinct appressorium before penetration. Appressorium formation by the stomatal-penetrating rusts is considered to be a recognition response evolved to ensure penetration at the correct site (Mendgen *et al.*, 1988; Read *et al.*, 1992). The orientated growth of the BYR germ tube implies that BYR is capable of perceiving topographical signals important for this response, but the long germ tubes suggest that sensing of topographical signals responsible for appressorium induction does not occur. The BYR germ tube often grew over one or more stomata before penetration occurred (chapter 3) whilst BBR usually differentiated an appressorium over the first stoma encountered (chapter 4). Given that penetration by BYR then occurs without the aid of an appressorium, it is clear that this organ is not an essential prerequisite for stomatal penetration by rusts. Energy will be expended during the formation of an appressorium and successful stomatal penetration without an appressorium may therefore represent an evolutionary progression or, at the very least, an alternative yet successful strategy. The absence of a distinct structure over the penetration site may be one reason why *P. striiformis* prefers a much lower temperature optimum than *P. hordei* and other cereal rusts for initial infection. The survival of the long BYR germ tubes would be more dependent upon favourable conditions of humidity than would the shorter germ tubes of BBR, and higher relative humidities in the field are very often associated with lower temperatures. Furthermore, appressoria are more resistant to desiccation and other unfavourable conditions, than are germ tubes (Emmett & Parbery, 1975). However, as might be expected from the semi-systemic type of

growth within the leaf, the frequency of germ tube penetration by *P. striiformis* is not critical and has been shown to have very little influence on subsequent pustule frequency (McGregor & Manners, 1985).

6.3.4. Vesicles

The morphology of vesicles and primary infection hyphae can be used to identify closely related rust species (Niks, 1986*b*; Opel & Lausch, 1987). The vesicle of BBR is elongated with a central septum and the primary infection hyphae typically possess a septum at the point of origin from the vesicle (chapter 4). This contrasts markedly with BYR which forms a globose, aseptate vesicle (chapter 3).

6.3.5. Invasive hyphae

The secondary infection hyphae of BBR (i.e. the hyphae developed from the bipolar primary infection hyphae) are always septate. They usually branch behind septa and quickly grow locally around mesophyll cells in which they form haustoria from terminal haustorial mother cells. The invasive runner hyphae of BYR (i.e. the hyphae developed from the primary infection hyphae) are large and aseptate, grow relatively long distances before branching, and often produce haustorial mother cells at mesophyll cell junctions along their length.

6.3.6. Colony spread after penetration

The delay by BYR in colony spread outwith the substomatal cavity suggests a period of establishment which may be necessary to permit an increase in nutrient levels to fuel the rapid invasion of leaf tissue beyond the substomatal cavity. This lag phase in the growth of BYR results in a longer generation time than that of BBR. Such a delay in establishment would be detrimental for BBR which relies upon re-infection for spread within the leaf tissue.

6.3.7. Colony growth pattern

The different colony growth patterns of the two rusts may be attributable in part to the morphologies of their respective invasive hyphae. Extensive growth into uninfected leaf tissue some distance away from the site of penetration is possibly the only way that the relatively unbranched, large invasive hyphae of BYR could obtain the maximum area of contact with host cells as would be necessary to sustain the colony. The smaller infection hyphae of BBR can fill all the intercellular spaces in a relatively smaller volume of the leaf.

Unlike BBR, BYR has no known alternate host thus relying upon other mechanisms for variation (Manners, 1988; Chen *et al.*, 1993). The extensive growth of BYR would increase the probability that hyphae from different infection sites would meet and fuse as has been previously observed between germ tubes of *P. striiformis* (Little & Manners, 1969). Within the yellow rust colony at this time the hyphae are aseptate and multinucleate (Allen, 1928, Wright & Lennard, 1978). Fusion between hyphae of compatible isolates could result in an exchange of nuclei, thus possibly providing a mechanism for genetic variation via nuclear re-assortment into dikaryotic urediniospores (Little & Manners, 1969).

6.3.8. Hyphal specialisation

The relatively large, aseptate runner hyphae of BYR produced thin, highly branched hyphae with terminal haustorial mother cells. These hyphae are involved in the formation of primary uredinial beds. Secondary uredinial beds of BYR, on the other hand, develop from thin spiky hyphae which are branches of the large septate runner hyphae. The invasive runner hyphae of BYR alone would possibly be too large to form the close network of hyphae around mesophyll cells which is necessary to maximise haustorial formation in these areas for reproduction. In contrast, apart from primary infection hyphae which are slightly larger than invasive hyphae, the BBR mycelium is composed of smaller, morphologically uniform hyphae which are apparently capable of both the invasive and reproductive phases of growth (sections 4.2.2 and 4.2.3). In the case of BYR, the first appearance of uredinial bed hyphae also signals the first appearance of a general septation within the colony which, amongst other roles, is probably important for cell specialisation during reproduction (section 3.3). *P. hordei*, on the other hand, has a more immediate investment in reproduction and, to this end, septation necessary for this is immediately in place. In contrast, *P. striiformis* seems to invest a greater period in seeking new nutrient resources within the leaf, as indicated by its semi-systemic growth behaviour, which might explain why septation is delayed until required.

6.3.9. Site of primary uredinium

The primary uredinium of BBR is always formed in the centre of the colony and is surrounded by mycelium. The reproductive phase is possibly triggered by a reduction in nutrient levels in the oldest part of the colony at the site of penetration. The primary uredinium of BYR is formed some distance from the penetration site by morphologically distinct hyphae branching from the initially

aseptate invasive runner hyphae. It is possible that nutrient starvation may also have a role in the induction of reproduction in this rust. Possibly the number of haustorial mother cells supplying nutrients to the invasive hyphae is important in this respect although this is pure speculation at present.

6.3.10. Pedicels

The length of the spore bearing pedicels within the BBR and BYR uredinia appears to influence the final shape of the uredinium. The BYR uredinia contain short pedicels and consequently are flatter than those of BBR which have long pedicels toward the centre of the uredinium (sections 3.3 and 4.3).

6.3.11. & 12. Secondary uredinia and period of spore production

BYR forms more uredinia per leaf than BBR and forms them over a longer time. The semi-systemic spread of BYR through the leaf results in uredinia along its length and only one successful penetration can be enough to infect the entire leaf (Sharp, 1965). The majority of secondary uredinia form towards the base of the leaf (section 3.2) possibly because, as in the case of multiple infections by *P. striiformis* f. sp. *tritici*, more penetrations occur in the mid and tip regions of the leaf (Russell, 1977). The spread into uninfected leaf tissue towards the base of the leaf means that there is an extended period of spore production as the leaf senesces from the tip. [In cereal leaves, the basal regions of the leaf are younger (Langer, 1972) and therefore would be able to support infection after the rest of the leaf has started to senesce.] In BBR, the area of spore production remains localised around individual penetration sites and the period of spore production is shorter. The prolonged presence of chlorophyll within the dense colonies of BBR (Scholes & Farrar, 1987) probably helps to sustain spore production by this rust. As senescence occurs in the uninfected leaf tissue around the colony, the infected host tissue can remain in a juvenile condition and support the secondary uredinia around the primary uredinium (section 4.3). Generally, the length of the period of spore production by rusts may be a survival feature: a long sporulation period may bridge periods where conditions are adverse to re-infection (Zadoks, 1971).

6.3.13. Environmental conditions for disease

BBR has a higher optimum temperature requirement than BYR (Table 2) and rapid disease development only occurs in warm summer weather, whereas most severe epidemics of yellow rust occur in temperate cool and wet climates (Rapilly, 1979). The cool temperate zones which suit barley (Langer & Hill, 1991) would seem to

favour the prolonged, and initially slower, 'systemic' development of infection by BYR as new plant tissue appears at the base of the leaf during emergence. BBR, on the other hand, is better suited to the higher temperature regime where infection development is more rapid.

Both rusts require moisture for germination and penetration and this is usually satisfied by the presence of dew. If the relative humidity is continuously below 100%, as in a hot dry summer, germination is prevented. In this case, BYR can uncharacteristically be more abundant than BBR at relatively high temperatures (Manners, 1982). This is because BBR relies upon secondary infections for spread and these require continuing periods of high humidity for germination. On the other hand, BYR is less dependent upon the maintenance of near-saturated conditions for germination because of its semi-systemic type of growth and this fungus can spread up to 10 times faster than BBR within the leaf (Manners, 1982). However, spores of BYR produced in warm, dry conditions are not as viable as those produced at low temperatures and high humidities (Gopalan & Manners, 1984). Both pathogens may appear in areas with favourable moisture levels and the predominance of a particular rust is ultimately temperature dependent. In hot dry weather, leaves infected by yellow rust become desiccated prematurely (Gair *et al.*, 1978). Urediniospores produced from a senescing leaf are of low viability (Gopalan & Manners, 1984). The fact that BYR is best adapted to lower temperatures is probably a major reason why this rust is not as prevalent as BBR, even though BYR has the more efficient infection strategy (section 5.3.5).

6.4. Comparison of BBR and BYR: final comments

It is extremely clear from this study that, although these two species are within the same genus, they differ markedly in their developmental morphologies and infection strategies on barley. Considering that *P. striiformis* does not need to produce an appressorium for infection (chapter 3), has the more efficient infection strategy (section 5.3.5) and produces variation without a sexual phase (Line, 1993), this rust might be considered more advanced than *P. hordei* and possibly other stomatal-penetrating cereal rusts.

In Scotland, where barley is still the main cereal grown (e.g. 66.7% of the total acreage of cereals in 1993, D. Cranstoun, pers. comm.), the last serious outbreak of barley yellow rust was in 1973 on spring barley (D. Cranstoun, pers. comm.). This was in a coastal area which is still one of the worst areas for wheat yellow rust. Yellow rust on spring barley in the 1970's was associated with inoculum that overwintered on highly susceptible winter barley cultivars (Gair *et*

al., 1978). This source of inoculum has largely disappeared with the demise of the cultivars Sonja and Athene (D. Cranstoun, pers. comm.). The absence of significant outbreaks of this disease on barley in the U.K. in recent years, may reflect the existence of a level of horizontal resistance that has possibly been inadvertently bred into some of the commercial barley cultivars grown at present (N. Simmonds, pers. comm.).

The small outbreaks of barley yellow rust that presently occur in Scotland are in the same coastal areas as the outbreaks of wheat yellow rust mentioned above, and have been associated with the occurrence of sea-mists (M. Richards, pers. comm.). This would agree with the finding that urediniospores of BYR are more viable when produced in conditions of high humidity (Gopalan & Manners, 1984). However, the majority of barley in Scotland is currently grown in the drier inland regions. Spring, rather than winter, barley cultivars still occupy the greater acreage (e.g. 227,000 ha for spring barley compared with 43,000 ha for winter barley in 1993, D. Cranstoun, pers. comm.) and in this case there would be nothing to infect until April. The absence of significant levels of disease suggests that the Scottish climate is unsuitable for yellow rust development at this time. In addition, the continual threat of mildew (caused by *Erysiphe graminis*) infection means that most barley crops are treated with fungicide. In summary, it may be that in recent years, suitable combinations of cultivar and environment have not occurred for the development of significant outbreaks of yellow rust on barley.

7. CONTACT SENSING IN *PUCCINIA GRAMINIS* F. SP. *TRITICI* AND *PUCCINIA HORDEI*

7.1. Introduction

In this chapter, the role of topographical signals in germ tube contact sensing has been investigated, with particular emphasis on appressorium formation. Two cereal rusts forming appressoria have been studied: *P. hordei* and *P. graminis* f. sp. *tritici* (wheat stem rust).

7.2. Results

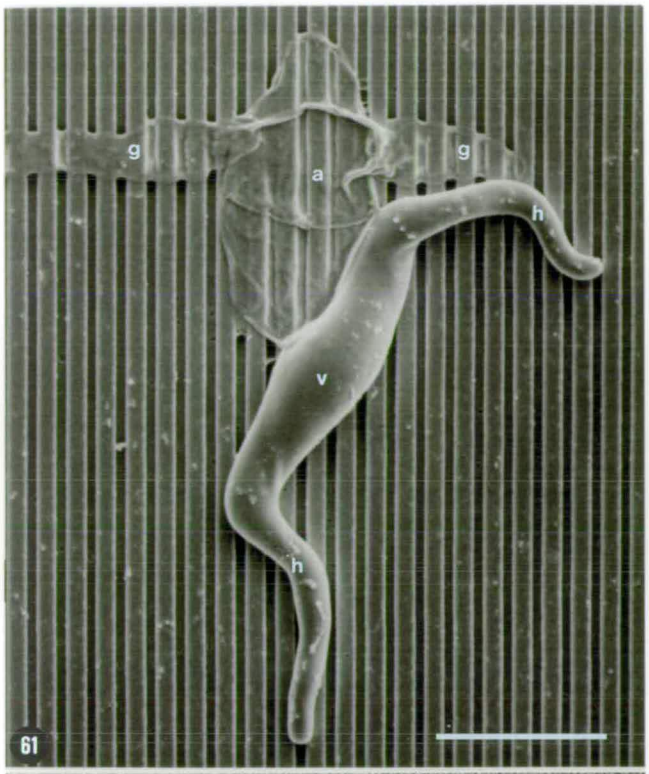
In vivo, the germ tubes of both *P. hordei* and *P. graminis* (not shown) exhibited marked directional growth which was approximately perpendicular to epidermal cell junctions. Short lateral branches were often formed by both species over the cell junctions. On encountering a stomatal complex, a germ tube differentiated an elongated appressorium which was specifically located over and covered the stomatal pore and most of the regions of guard cells adjacent to the pore (Fig. 32 in chapter 4; Fig. 62). Penetration through the pore occurred after appressorium development and typically resulted in a succession of differentiation events within the substomatal cavity. The series of infection structures formed by both species were similar and comprised of: an infection peg; an elongated, septate vesicle; infection hyphae arising at two ends of the vesicle; and a haustorial mother cell forming at the end of each infection hypha on contact with a mesophyll cell surrounding the substomatal cavity (Fig. 2 in chapter 1; Fig. 33 in chapter 4). In susceptible host-pathogen combinations, the haustorial mother cell normally gave rise to an intracellular haustorium (Fig. 40 in chapter 4).

In vitro on flat polystyrene surfaces, 0.77% (s.e. 0.23%) of germ tubes of *P. hordei* and 1.3% (s.e. 0.28%) of germ tubes of *P. graminis* differentiated appressoria. 1.04% (s.e. 0.23%) of germ tubes of *P. hordei* and 1.64% (s.e. 0.32%) of germ tubes of *P. graminis* differentiated appressoria on single ridges (2 μm wide) with heights varying from 0.116 - 2.4 μm. Differentiation on single ridges was not significantly different from that on flat surfaces. However, significantly greater differentiation was induced when the two species were grown on closely spaced, multiple ridges (e.g. see Fig. 61 for *P. hordei*). The extent of

Fig. 61. Scanning electron micrograph of infection structure differentiation of *P. hordei* on a wafer replica with closely spaced ridges. Note: the collapsed germ tube (g) which grew perpendicular to the ridges; the appressorium (a) formed along the ridges. The turgid vesicle (v) and bipolar primary infection hyphae (h) are infection structures which would normally be formed within the substomatal cavity of the host. Partially freeze-dried. Bar = 20 μm.

Fig. 62. Scanning electron micrograph of appressorium (a) differentiation of *P. hordei* over a stomatal complex. The collapsed germ tube (g) has grown across the epidermal cell wall junction where it has produced a lateral branch (arrow). Note the appressorial lobes (l) over the cell wall junctions of the guard cells and the companion cells. Partially freeze-dried. Bar = 20 μm.

Fig. 63. Scanning electron micrograph of the stomatal complex of a barley leaf showing the companion cells (c) and the dumb-bell shaped guard cells (g). Note that the guard cells do not possess prominent lips adjacent to the stomatal pore. Partially freeze-dried. Bar = 10 μm.



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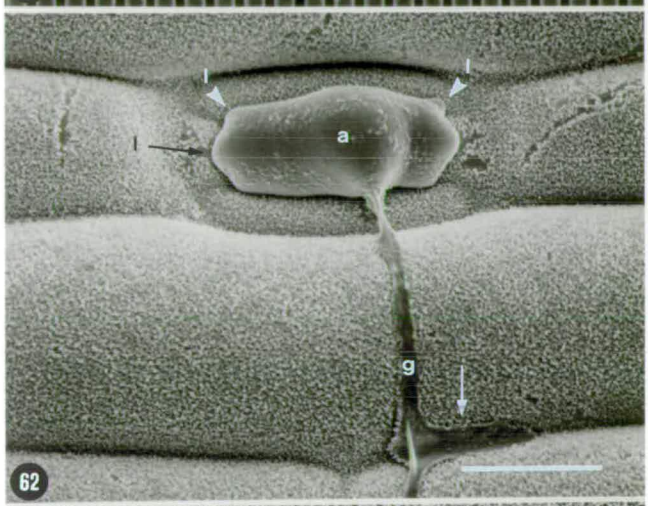

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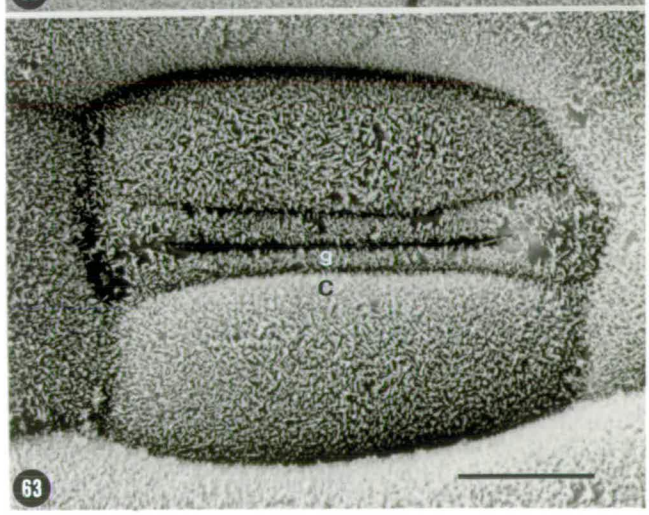

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differentiation depended on the precise spacing of the ridges and their heights (Figs. 64, 65). For both species the most inductive ridge spacing was consistently 1.5 μm for virtually all the heights analysed, and the greatest differentiation was recorded over ridges which were 2.0 μm high (83% and 86% respectively for *P. hordei* and *P. graminis*, Figs. 64*a*, 65*a*).

The influence of ridge spacings and heights on the extent of infection structure differentiation beyond appressorium formation was also assessed (Figs. 64*a,c* and 65*a,c*). At the 2.0 μm ridge height and 1.5 μm spacing, found to be most inductive for the initiation of differentiation, 82.2% of the total number of *P. hordei* germ tubes which differentiated (i.e. taking total differentiation as 100%), formed infection structures beyond appressoria. Fewer germ tubes (37.7%) of *P. graminis* differentiated beyond the appressorial stage. However, it was notable that at the wider ridge spacing (2.5 μm), a greater number (69.1%) of *P. graminis* germ tubes differentiated beyond appressoria.

It was found that germ tubes grew to variable lengths before differentiating on the topographies bearing closely spaced ridges or troughs. When they grew over more than a few of these ridges and troughs, the growth pattern of the germ tubes was markedly perpendicular to the orientation of the substratum topography (Fig. 61).

Differentiation over topographies with different numbers of ridges (2.0 μm high and 2.0 μm wide) and 2.0 μm troughs was quantified and the influence of the following four topographies compared: (a) one ridge, no trough; (b) two ridges, one trough; (c) three ridges, two troughs; and (d) four ridges, three troughs. At the 2.0 μm ridge height, found to give the greatest overall differentiation (Figs. 64, 65), the differentiation over a single ridge was 3.31% (s.e. 0.42%) by *P. graminis* and 1.43% (s.e. 0.77%) for *P. hordei* (Fig. 66). The mean differentiation of each species over two closely spaced ridges was 7.13% for *P. graminis* and 6.54% for *P. hordei* which were both significantly greater ($P \leq 0.05$) than the differentiation on single ridges at this height. Differentiation increased significantly ($P \leq 0.05$) from 2 to 4 ridges (Fig. 66). The maximum differentiation over 4 ridges was 18.62% (s.e. 2.62%) for *P. hordei* and 10.88% (s.e. 1.49%) for *P. graminis*.

Examination of the spacing of cell junctions across the surfaces of the mid region of seedling leaves of barley demonstrated that the spacings between epidermal cells was great (26 - 61 μm apart, $n = 40$). This contrasted markedly with the narrow widths of the dumb-bell shaped guard cells which, in the region immediately adjacent to the stomatal pore, varied from 1.79 - 4.71 μm ($n = 40$).

Figs. 64, 65. Differentiation of *P. hordei* and *P. graminis* f. sp. *tritici* on wafer replicas. Mean ± s.e.

Fig. 64. Infection structure differentiation by *P. hordei* over ridges of different heights and spacings.

Fig. 64*a*. Percentage of germ tubes which differentiated.

Fig. 64*b*. Percentage of germ tubes which differentiated appressoria only.

Fig. 64*c*. Percentage of germ tubes which differentiated appressoria and vesicles.

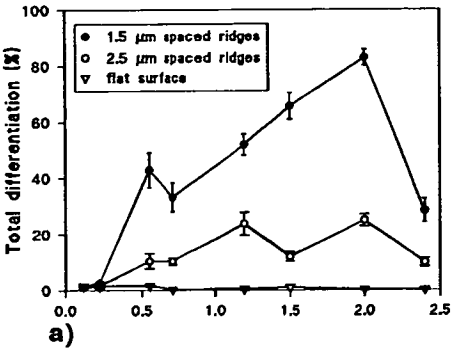
Fig. 65. Infection structure differentiation by *P. graminis* f. sp. *tritici* over ridges of different heights and spacings.

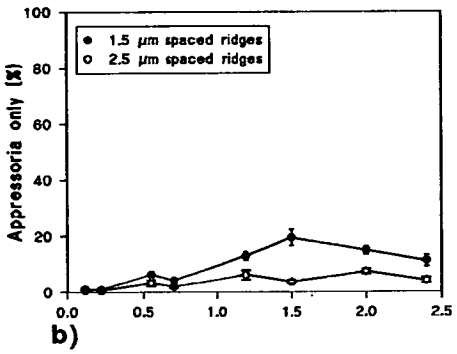
Fig. 65*a*. Percentage of germ tubes which differentiated.

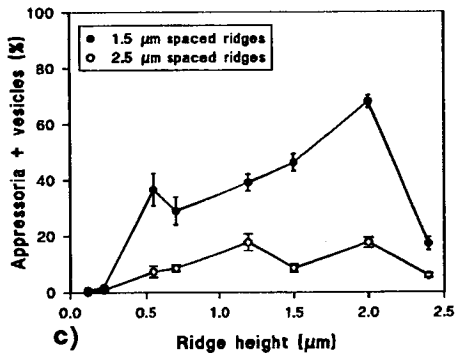
Fig. 65*b* Percentage of germ tubes which differentiated appressoria only.

Fig. 65*c* Percentage of germ tubes which differentiated appressoria and vesicles.

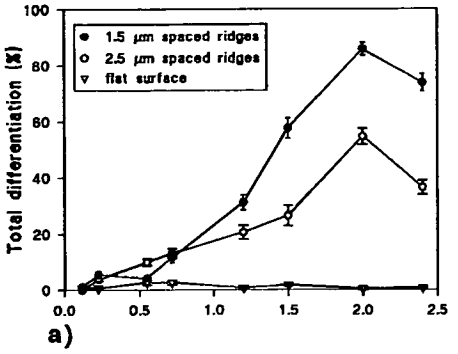
P. hordei

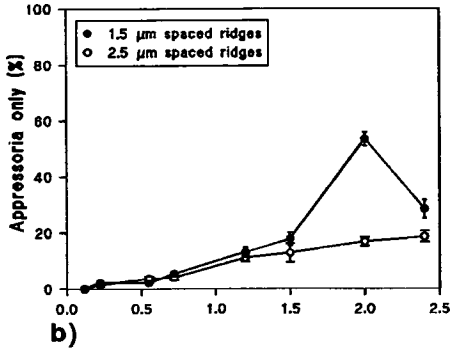


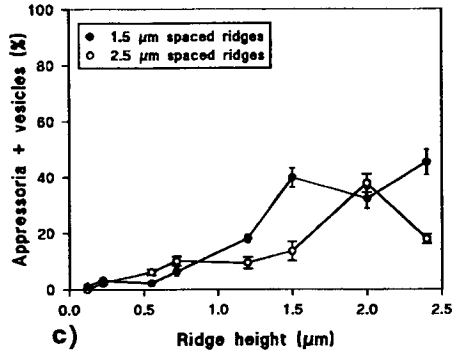




P. graminis







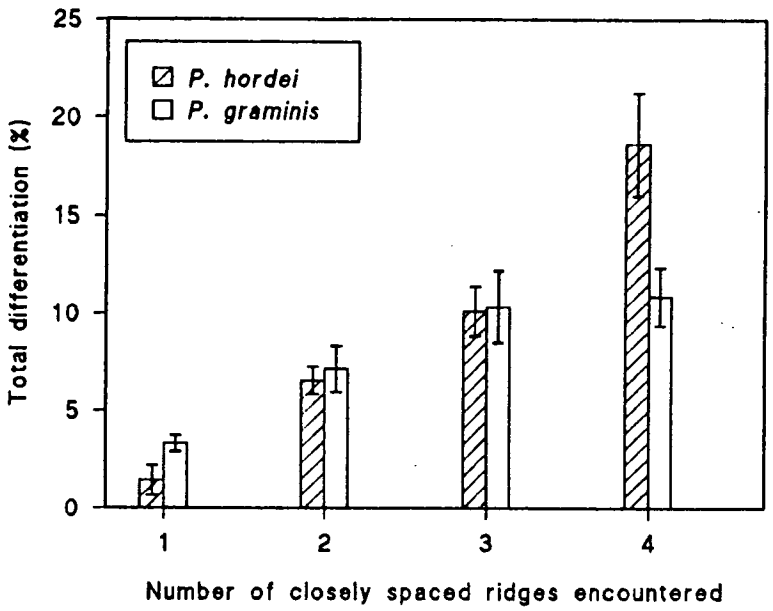


Fig. 66. Differentiation of *P. hordei* and *P. graminis* f. sp. *tritici* over different numbers of closely spaced 2.0 μm high ridges on wafer replicas. Mean $\pm$ s.e.

The latter analysis involved measurements on guard cells with associated pores of variable aperture. The widths of companion cells was found to vary from 5.27 - 15.02 μm (n = 40). Another notable observation regarding potential topographical signals was that the guard cells lacked prominent lips adjacent to their associated pores (Fig. 63).

7.3 Discussion

It has been shown here that two different cereal rusts can be induced to differentiate appressoria and subsequent infection structures by topographical features alone. A close spacing of ridges and/or grooves was found to provide the necessary inductive topographies which resulted in 83-86% of germ tubes differentiating appressoria. The stomatal complexes of cereal leaves do not possess prominent guard cell lips which are believed to provide inductive stimuli for appressorium formation in many rusts which infect dicotyledonous hosts (Terhune *et al.*, 1991; Wynn, 1976; Hoch *et al.*, 1987; Allen *et al.*, 1991*a,b*; Read *et al.*, 1992). In contrast to these species, it is suggested here that the two cereal rusts described in this thesis, and possibly other cereal rusts as well, respond to the close spacing of cell junctions associated with the dumb bell-shaped guard cells of cereal leaves. Measurements of the spacings of these cell junctions showed them to be 1.79-4.71 μm which overlapped with the spacing (1.5-2.5 μm) of the inductive artificial topographies used here.

It seems likely that the precise topographical features which induce appressorium formation *in vivo* have not been identified in this study. One reason for this is that appressoria are not formed over the first closely spaced topographical features which they encounter when grown over numerous, closely spaced ridges. This was further demonstrated by a significantly reduced differentiation (< 18.6%) over topographies with 2-4 ridges. In addition, the percentage of germ tubes which differentiated into appressoria was not as great as that which occurs when germ tubes encounter stomatal complexes *in vivo* (97% for *P. hordei* and 90% for *P. graminis* f. sp. *tritici*, Collins, T. and Read, N. D., unpublished). To determine the inductive topographies operating *in vivo*, analysis of the precise topographies associated with cereal stomatal complexes will be required.

Another consideration is that chemical factors associated with the stomatal complex may provide additional signals which increase the number of appressoria

which differentiate *in vivo*. Both volatile and non-volatile cuticular components have been isolated from wheat leaves and found to induce differentiation of *P. graminis* f. sp. *tritici in vitro* (Grambow & Reisener, 1976; Grambow 1977, 1978; Grambow & Riedel 1977; Grambow and Grambow 1978). It may be that by being able to use either topographical or chemical signals, or better a combination of the two, *P. graminis* f. sp. *tritici* and other cereal rusts can maximise appressorium differentiation over stomata. However, inductive biochemical domains located specifically around stomata remain to be demonstrated.

In this study, the topographies which induced appressorium differentiation also often initiated a cascade of differentiation events involving the formation of a succession of infection structures up to the stage of infection hyphae. Haustorial mother cells were rarely formed. This suggests that once appressorium formation has been induced then the fungus becomes committed to a programme of differentiation up to the infection hypha stage. Haustorial mother cell differentiation may require further contact and/or chemical signals to be optimally induced. In contrast to the results reported in this thesis, Staples *et al.*, (1983) found that vesicles and infection hyphae were not formed when appressoria of *P. graminis* f. sp. *tritici* were induced on scratched membranes. The reason for this discrepancy between the two studies is not clear but may relate to either different races of the fungus used or the superior inductive topography employed in the work reported in this thesis.

A feature of rust urediniospore germ tubes is that they exhibit directional growth which is more-or-less at right angles to epidermal cell junctions (anticlinal cell walls). This growth pattern on cereal leaves, in which the stomata are arranged in staggered, parallel longitudinal rows, seems to maximise the chances of a germ tube encountering a stoma (Johnson, 1934; Read *et al.*, 1992). It has been suggested that a regular lattice of wax crystals on wheat leaf cuticles provides the guidance cues for directional growth of *P. graminis* f. sp. *tritici* (Lewis and Day 1972). This now seems unlikely since germ tubes of *P. graminis* f. sp. *tritici* and *P. hordei* exhibit marked directional growth on scratched and microfabricated, artificial substrata in the absence of wax crystals, as shown previously (Staples *et al.*, 1983; Read *et al.*, 1992) and in this study.

8. FUTURE WORK

Below is a list of important studies following on from the research reported in this thesis which, given the opportunity, I would have done.

1. Survey the responsiveness of all the main rusts of wheat, barley, oats and rye to multiple and single ridges.

2. Determine the influence of topographical and chemical signals on appressorium induction by combining them *in vitro*.

3. Analyse precise topographies of cereal leaves which induce appressorium formation of BBR and WSR *in vivo*.

4. Determine whether mitosis is induced in germ tubes of *P. striiformis* when they encounter stomata (mitotic nuclear division is a feature of appressorium formation).

5. Determine whether topographical signals can induce vesicle formation in *P. striiformis*.

6. Determine whether appressorium formation by *P. hordei* is influenced by guard cell size, and thus cell junction spacing, on barley leaves (different ploidy levels of barley are available which differ in their guard cell size).

7. Determine whether directional growth and/or appressorium formation of BYR, BBR and WSR can be inhibited by coating leaf surfaces with film-forming polymers (Zekaria-Oren & Eyal, 1991).

8. Determine whether vesicles of *P. striiformis* can be induced to form on the leaf surface and on microfabricated topographies.

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